Bone Ash Data

in the context of phosphorus and phytase evaluation in poultry

Susanne Künzel

Institute of Animal Science
Department of Animal Nutrition
Prof. Dr. Markus Rodehutscord

BONE ASH DATA IN THE CONTEXT OF PHOSPHORUS AND PHYTASE EVALUATION IN POULTRY

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3. Prüfer:	Prof. Dr. J. Bennewitz

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LIST OF ABBREVIATIONS

Abbreviations for units defined by the international system of units and abbreviations exclusively used in the included manuscripts or tables are not listed here.

ANOVA	Analysis of variance
BW	Body weight
Ca	Calcium
DCP	Dicalcium phosphate
DM	Dry matter
$InsP_6$	*Myo*-inositol1,2,3,4,5,6-hexakis(dihydrogenphosphate)
MI	*Myo*-inositol
N	Natuphos® 5000 G
NE	Natuphos® E 5000 G
P	Phosphorus
pcdP	Precaecally digestible phosphorus
PU	Phosphorus utilisation
QTL	Quantitative trait locus
R	Pearson correlation coefficient
R^2	Coefficient of determination
rP	Retained phosphorus
Tibia	Tibiotarsus
WPSA	World´s Poultry Science Association

1 GENERAL INTRODUCTION

For 2018, the Food and Agriculture Organization of the United Nations (2020) reported more than 25.6 billion living poultry birds in the world. Since 1961, when 4.4 billion poultry birds were counted, this number increased continuously, and it is expected to further rise in the next years. Poultry play an important role in human nutrition in all parts of the world by providing meat and eggs, in developing countries as well as in industrialised nations.

Poultry need to be fed adequately to ensure animal wellbeing, safe and nutrient-rich food products and to protect the environment. Providing animals with phosphorus (P) is an important part of poultry nutrition. It is an essential element for all organisms since it is needed for numerous physiological processes like energy metabolism, nucleic acid synthesis, and bone mineralisation. The main components of poultry diets are plant seeds, where P is mainly stored in the form of *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogenphosphate) ($InsP_6$) and its salts (phytate) (Eeckhout and De Paepe, 1994; Rodehutscord et al., 2016). This form is only partially available to poultry. Therefore, P is often supplemented to poultry diets as mineral P mined from rock phosphate and processed. However, global rock phosphate reserves are limited. Li et al. (2018) predicted depletion of these reserves within 70-140 years when no appropriate management procedures are implemented. With a suitable P management, this time frame can be extended by 50 years or more according to their demand-based calculations. A suitable P management is also important to reduce environmental impact. A high P excretion due to a low digestibility results in an accumulation of P in the soil, which is a cause for eutrophication when stocking density is high (Schindler, 1977; Carpenter et al., 1998).

Phytate degrading enzymes (phytases) are widely used as feed additives in poultry diets. Phytases do naturally occur in plants and animals. In broilers, various studies have shown a remarkable precaecal degradation of $InsP_6$ (56-89 %) when low P diets without phytase supplementation were fed (Leytem et al., 2008; Zeller et al., 2015a; Zeller et al., 2015b; Sommerfeld et al., 2018). This could be related to some of the microorganism species frequently occurring in the gastrointestinal tract of broilers. They were identified to have phytase-like activity, most of them belonging to the genus *Lactobacillus* (Sümengen et al., 2012; Lee et al., 2013; Sumengen et al., 2013; Amritha et al., 2017). A study with gnotobiotic birds, however, has also shown a remarkable $InsP_6$ degradation of 42 % until the end of the ileum without phytase supplementation (Sommerfeld et al., 2019). This points towards the existence of endogenous mucosal phytase activity. Commercially available exogenous phytase products are fungi- or bacteria-derived. They have a specific spectrum of activity since they differ in their optimum pH, thermal stability and they start dephosphorylating

the phosphate either at position 3 or 6 of the *myo*-inositol hexaphosphate ring (Konietzny and Greiner, 2002).

To increase P utilisation of the animal and to protect the environment, it is necessary to know all physiological processes which involve P and to have suitable approaches for the determination of available P in the animal. This is necessary since plant-based feedstuffs contain different amounts of phytate-P and intrinsic phytase. Other constituents of the feed and feed additives also have an impact on physiological processes in the animal, probably influencing $InsP_6$ degradation. Hence, it is difficult to predict the availability of P for different P sources. A protocol established by the World´s Poultry Science Association (WPSA, 2013) provides a standard procedure for the determination of available P in broilers. It involves regression analysis with precaecally digestible P (pcdP) as the response trait. Consequently, the collection of digesta from the ileum and their analysis are necessary. For comparative purposes, the use of bone data or other biological data such as body weight gain or blood inorganic phosphate concentration have been used to represent the relative bioavailability of P (Shastak and Rodehutscord, 2013).

Biological response traits are influenced by the amount of available P provided. Bones are the preferred tissue because 80 % of the P retained in the animal is stored therein (De Groote and Huyghebaert, 1997). Tibiotarsus (tibia) and femur bones of 40-day-old Ross 308 broilers consist of approximately 50 % ash, 40 % organic matter, which is mostly in the form of collagen, and 10 % water (McLean, 1958; Suchý et al., 2009). Suchý et al. (2009) analysed 20 % of bone dry matter (DM) to be calcium (Ca) and 9 % P. The major bone minerals are P and Ca and they are mainly stored in the form of hydroxyapatite (McLean, 1958). Accordingly, bones may be an appropriate indicator for the relative bioavailability of P. However, they are also influenced by other factors. Rath et al. (2000) reported the bone to be a dynamic tissue which is not only influenced by nutritional, but also by physiological factors. In humans, about 80 % of bone mineral density is genetically determined (Nguyen et al., 1998). This should be considered when using bone data for P evaluation.

At least since the 1940s, bone ash data have been used as an indicator of relative bioavailability of P (Bird and Caskey, 1943). However, a standard assay has never been agreed on. While most of the studies examined the tibia, others used the femur, one or more toes or the foot. Additionally, bones are used with or without ether extraction or other pre-treatments. When ash data are used, some authors analysed the P concentration in ash while others used the total amount of ash. Other options for using bone traits are the determination of bone breaking strength or densitometry (Shastak and Rodehutscord, 2013). It is not clear to what extent the assay details and chosen bone may affect the outcome of P bioavailability studies.

2 OVERVIEW AND OBJECTIVES OF THE INCLUDED STUDIES

The overall aim of this thesis was to investigate the suitability of bone ash data for the evaluation of available P in poultry with emphasis on broiler chickens. Therefore, different studies with broiler chickens and Japanese quail as model organisms for broilers were conducted. The studies formed the basis of four manuscripts which are presented in Chapter 4 and briefly characterised in the following. The studies had in common that bone ash data were used for the examination of the relative bioavailability of P. However, other aspects related to P availability in poultry were also studied. These data, together with additional unpublished data that accrued during the studies, were focused in this thesis with additional analyses. Especially methodological aspects were examined.

Phytase products and mineral P are commonly used feed supplements to provide poultry with available P in the adequate amount. Each product has a specific efficiency, and they can only be compared under standardised study conditions. The influence of two different phytase products and dicalcium phosphate (DCP) as a mineral P source on available P and the ileal microbiota of broiler chickens was investigated in the first study (**MANUSCRIPT 1**). Each of the three supplements was fed in graded inclusion levels in addition to a low P basal diet without any phytase or the mineral P supplement. This design allowed to compare the supplements with a regression approach, as suggested by the WPSA (2013). Several response traits for P evaluation were used and compared. Besides the commonly used standard trait for quantitative purposes (pcdP) also other traits such as average daily gain or tibia and foot ash, as both absolute amount and concentration were examined.

When $InsP_6$ is completely dephosphorylated, each of the six phosphate groups and the *myo*-inositol (MI) ring are potentially available for the bird. Recent studies have shown that the supplementation of phytase to the feed increased the MI concentration in digesta or excreta of broiler chickens, thus complete dephosphorylation seems possible (Beeson et al., 2017; Sommerfeld et al., 2018). While most of the effects of the phosphate groups on the animal are identified, MI still needs more detailed investigations. The study described in **MANUSCRIPT 2** aimed to consider the effects of free MI in the diet on performance, nutrient digestibility, $InsP_6$ breakdown and MI concentrations in the digestive tract and blood plasma of broiler chickens. Bone mineralisation was analysed with the amount of tibia ash to detect the bioavailability of P. MI supplementation was compared with a control diet adequate in all nutrients and three treatments with different supplementation levels of a phytase product.

Broiler chickens have a high potential to degrade $InsP_6$ when low P diets are fed without supplementation of phytase (Zeller et al., 2015a; Zeller et al., 2015b; Sommerfeld et al., 2018). This

is indicative for endogenous phytase activity originating from epithelial tissue or the gut microbiota (Rodehutscord and Rosenfelder, 2016). Endogenous phytase activity might be affected by the genome of the animal. Hence the selection of animals with a high potential for P utilisation (PU) seems desirable. Previous studies have shown a moderate heritability of PU by quails (0.136; Beck et al., 2016a) and broiler chickens (0.10; Zhang et al., 2003). Since PU is difficult to determine under practical conditions, the study described in **MANUSCRIPT 3** aimed to examine the potential of bone ash data for P breeding with quantitative genetic analyses. Therefore, tibia and foot ash of Japanese quail were used as amount and concentration and compared as criteria of bone mineralisation and in genetic analysis.

Results of a P ring test have shown that precaecal P digestibility and $InsP_6$ breakdown varied to a large extent between different experimental stations, even when the same experimental diets were used (Rodehutscord et al., 2017). However, starter diets were provided by each station individually and some but not all starter diets were supplemented with a coccidiostat. Coccidiostats are a group of different agents approved as feed additives for the prevention of coccidiosis in the EU. They are known to affect the gastrointestinal microbiota. It is also known that some microorganisms in the gastrointestinal tract of poultry can produce phytase and consequently can affect $InsP_6$ breakdown. The study which resulted in **MANUSCRIPT 4** investigated the influence of a widely used coccidiostat product on $InsP_6$ breakdown and the gastrointestinal microbiota in broiler chickens at different P, Ca and phytase levels. Bioavailability of P was investigated with the determination of foot ash data.

3 GENERAL DISCUSSION

3.1 Comparison of tibia and foot ash

Bones are used for the evaluation of relative P bioavailability because a high amount of P in the body is stored there. The development of bones in chicken varies between different body parts (Church and Johnson, 1964; Lilburn, 1994). Therefore, it seems unlikely that P is evenly distributed among different bones. This leads to the conclusion that some bones may be more suitable for P evaluation measurements than others. Additionally, some bones are easier to obtain than others because of their localisation in the body. For example, toe bones are easy to obtain and because of their small size, they are fast to analyse since they need less time to be incinerated than bigger bones. However, while some authors recommend using toe ash, others reported a high variation between animals of one treatment for toe ash because small variations in sampling technique have a high impact on the low ash weight (Scholey and Burton, 2017). The animal age is also of importance for the decision which bone is preferred. Scholey and Burton (2017) compared toe, foot, tibia and femur ash of broiler chickens as response traits of bone mineralisation at weeks 2-6 of age. Toe bones showed only in week 2 a difference between an adequate and a low P diet, femurs in weeks 4-6, tibiae in week 2-6 and feet in week 2-5. Most of the P evaluation studies using broiler chickens end after 20-30 d. Based on the results of Scholey and Burton (2017), tibiae and feet are the most suitable bones for this animal age. Indeed, the tibia is the most often used bone for this kind of analysis and there is some evidence in the literature that foot ash could be used equivalently (Mendez and Dale, 1998; Yan et al., 2005; Malloy et al., 2017). Therefore, tibia or foot ash or both were used for the studies resulting in Manuscripts 1-4 and these bones will be focused on in the following.

Many authors used lipid extracted bones for P evaluation studies like suggested as a standard method for vitamin D determination in bones (Association of Official Analytical Chemists, 1990). This was not done for the studies included here since there is strong evidence that lipid extraction does not provide beneficial effects regarding the sensitivity of the ash assay (Yan et al., 2005; Garcia and Dale, 2006). The used tibiae of the included studies were defrosted, adhering tissues removed, rinsed with distilled water, the bone dried to mass constancy for DM determination at 103 °C and then incinerated at 600 °C. Feet were cut at the *articulatio intertarsalis*, then also rinsed with distilled water, dried for DM determination, and incinerated at 600 °C. Therefore, the foot sample consists of multiple bones and tissues and has a higher mass than the tibia. It needs more time for drying and incinerating the feet compared to the tibiae, but it is much less laborious to detach them. The tibia has the advantage over the foot in being better standardised since one bone is a clearly defined

material. However, the fibula and the cartilage tissue, especially at the tibiofemoral joint, are sometimes difficult to remove. The tibia can be damaged in this process, leading to missing bone fragments. This is not a problem for foot samples. The tarsometatarsus can be detached from the tibia very well. In the case of the feet, the remaining soft tissues can possibly lead to sampling inaccuracy because it is hardly possible to standardise the segregation of skin. Consequently, the risk of inaccuracy when sampling feet is related to DM weight but not to bone mineralisation since the bones are still protected by the surrounding tissue.

3.1.1 Relationship between tibia and foot ash

The relationship between tibia and foot ash reported in the literature and determined during the own studies are shown in Table 1. Most studies showed a very high relationship between the two bone fractions with a coefficient of determination (R^2) between 0.63 and 0.96, indicating that they can be used almost equivalently. The highest relationship ($R^2 = 0.96$) was observed by Shastak et al. (2012a), the only study where bones without adhering tissues were analysed for foot ash. This may mean that the ash amount of the adhering tissues has a negative effect on the relationship between tibia and foot ash. Only in two studies a determination coefficient below 0.5 was detected. In the study of Scholey and Burton (2017; $R^2 = 0.46$), this could be related to a relatively small animal number which was distributed to different sampling times. The other study with a lower relationship ($R^2 = 0.47$) is described in Manuscript 3 and used ash concentration values of 15-day-old Japanese quail. The absolute amount of ash values of the same study provided a much higher relationship with $R^2 = 0.82$. The difference between the amount and concentration values of ash will be discussed in Chapter 3.3. In general, the relationship values between the two bone fractions in the study described in Manuscript 3 are lower than in most of the other studies. One explanation for the lower relationship is the animal age of only 15 d. Until the age of 21 d, bone legs of broilers grow faster compared with an age of 22-42 d (Han et al., 2015). The bone development including the ash amount differs between the tibia and the tarsometatarsus (Church and Johnson, 1964; Han et al., 2015), the major foot bone. The ash amount in soft tissues also changes during growth. Grey et al. (1983) observed a decreasing ash and P concentration in skin and different muscles with increasing age. Hence, the relationship between tibia and foot ash is supposed to be not constant, especially during the period of fast growth. Another possible explanation is the poultry species. Rodehutscord and Dieckmann (2005) compared different poultry species concerning their response in PU to a mineral supplement. They concluded that quail could be used as model organisms for broilers in P availability studies. However, there are no studies comparing the bone development of broilers and quail and the response of their bones to supplemental P. A comparison between quail and broilers in a combined trial using different P levels in the diet,

analysing bone ash and pcdP would be necessary to make sure there are no species-specific effects on bone ash data for the purpose of P evaluation.

Table 1: Relationship between tibia and foot ash observed in the literature and the own work with the coefficient of determination (R^2)

Reference	Used part of the foot[1]	Animal	Animal age (d)	Unit[2]	n	R^2
Mendez and Dale (1998)	n.d.	broiler	18	%	30	0.85
Yan et al. (2005)	whole foot	broiler	21	%	450	0.92
Shastak et al. (2012a)	bones	broiler	21	g	224	0.96
Shastak et al. (2012a)	bones	broiler	35	g	224	0.94
Malloy et al. (2017)	whole foot	broiler	21	%	240	>0.90
Malloy et al. (2017)	whole foot	broiler	43	%	384	>0.90
Scholey and Burton (2017)	whole foot	broiler	14-42	%	144	0.46
Manuscript 1	whole foot	broiler	21/22	mg	264	0.87
Manuscript 1	whole foot	broiler	21/22	%	264	0.63
Manuscript 3	whole foot	quail	15	mg	887	0.82
Manuscript 3	whole foot	quail	15	%	887	0.47

[1] n.d. = not described; whole foot = the foot was detached at the *articulatio intertarsalis* and used including skin, soft tissues and claws

[2] g or mg = absolute amount of ash weight; % = ash concentration related to the dry matter weight of the respective bone fraction

3.1.2 Phosphorus concentration in tibia and foot ash

The generally very high relationship between tibia and foot ash gives no prediction about which trait is more suitable for the purpose of P evaluation. For this information, the relationship between bone ash and P must be looked at more closely. Usually, only the ash weight is determined for P evaluation studies. During the study described in Manuscript 4, additional analyses were conducted to compare the P and Ca concentration in tibia and foot ash. Animals for this comparison were fed a low P and Ca diet without coccidiostat supplementation, either with or without phytase supplementation. The birds were allocated to six pens with ten birds each per treatment and sorted by their body weight (BW) after slaughter. They were distributed to three BW categories: light (3 birds), middle (4 birds) and heavy (3 birds). One bird per pen and BW category was randomly chosen for further ash analyses. The right tibia and foot were dried and incinerated as described above. Then P and Ca in the ash were analysed using the method described by Shastak et al. (2012b). Thereby the entire ash of each foot or tibia was used. Statistical analysis was done with a

two-way analysis of variance (ANOVA) using the PROC MIXED procedure of SAS 9.4 for Windows (SAS Institute Inc., Cary, NC). Treatment, bone fraction and the interaction between treatment and bone fraction were taken as fixed effects, the pen and animal as random effects.

Table 2: P and Ca concentration in tibia and foot ash and the ash weight of both bone fractions analysed in male 24/25-day-old broiler chickens[1]

	Phy-		**Phy+**		*pooled*	***p*-values[3]**		
	Tibia	**Foot**	**Tibia**	**Foot**	***SEM*[2]**	**Trt**	**BF**	**Trt × BF**
P g/kg ash	167	158	177	169	*0.72*	<0.001	<0.001	0.085
Ca g/kg ash	336[b]	300[d]	345[a]	317[c]	*1.71*	<0.001	<0.001	0.024
Ca:P	2.01[a]	1.91[c]	1.95[b]	1.88[d]	*0.009*	<0.001	<0.001	0.038
Ash mg	483	568	968	1057	*31.3*	<0.001	<0.001	0.831

[1] All birds were part of the study described in Manuscript 4 and received a P/Ca reduced diet without coccidiostat, either without (Phy-, n = 18) or with phytase supplementation (Phy+, n = 18)

[2] SEM = standard error of the mean

[3] Trt = Treatment (Phy- or Phy+), BF = Bone fraction (tibia or foot)

[a–d] Means within a line not showing a common superscript are significantly different (α = 0.05)

Results of this observation indicated a significantly higher P and Ca concentration in tibia ash compared to foot ash, both with and without phytase supplementation (Table 2). Phytase supplementation increased the concentration of P and Ca in both bone fractions significantly. The proportion of the two minerals also varied significantly between bone fractions and treatments, with the tibia and the phytase supplemented treatments resulting in the higher Ca:P ratio. This is basically in agreement with Han et al. (2015). They reported a higher P and Ca concentration in the tibia compared with the tarsometatarsus until the age of 35 d in broiler chickens. At the age of 42 d, they reported the tarsometatarsus to contain more P than the tibia. The whole foot was not analysed by these authors or any other study investigating the P concentration in ash. These results may lead to the conclusion that the tibia is the more suitable bone fraction for P evaluation studies until the age of 35 d since it contains more P than the foot. However, differences between the P concentration in tibia and foot are significant, though numerically very low. A higher ash weight of one bone fraction could have a higher impact than the P concentration. In the comparison done with broilers from Manuscript 4, the higher ash weight was detected for the foot ash compared to tibia ash, both with and without phytase supplementation (Table 2). When calculating the P concentration of each bone fraction, the foot contained 90 mg P/foot without and 179 mg P/foot with phytase supplementation. For the tibia, 81 mg P/bone without and 171 mg P/bone with phytase supplementation were determined. Consequently, the foot contains a higher amount of P. However, not the highest amount of P is necessary to detect the bioavailability of P, but the one

which represents best the P situation in the whole body. Shastak et al. (2012b) compared the tibia P content with the whole-body P. They assessed the tibia P to represent whole-body P adequately, but foot ash was not used in this study. The previously described results indicate that the difference between tibia and foot P is not very big. It seems possible that the slightly higher P concentration in feet even better represents the whole-body P. An experiment using both tibia and foot ash would be necessary to examine this in more depth.

3.1.3 Relationship between bone ash and quantitative traits of phosphorus evaluation

Another possibility to compare tibia and foot ash is to investigate their relationship with quantitative P evaluation traits. Therefore, Pearson correlation coefficients (R) were calculated for each of the own studies to compare tibia and foot ash as amount or concentration values with the content of pcdP, precaecal P digestibility and retained P (rP; Table 3). Highest correlation coefficients for almost all studies and traits were detected for the relationship of ash traits with rP. In contrast to pcdP and precaecal P digestibility, not only the P digestibility but also the feed intake and hence the actual P intake is considered for rP. Ash data reflect the combined effect of P intake and P digestibility during the whole experiment. Therefore, rP is more suitable than pcdP or precaecal P digestibility to be compared with ash data. Precaecal P digestibility showed no significant correlation coefficients with ash data of study 1 and 2. For study 4, the correlations were much smaller than for pcdP and rP. Precaecal P digestibility only refers to the P concentration in the feed, not the P intake, and consequently, it only reflects the situation in the animal at the time of slaughter. The trait pcdP is more standardised since it is related to 1 kg DM of the feed.

For Manuscript 2, almost all calculated correlation coefficients were not significant. In this study, the experimental diets of all treatments contained adequate P levels. Hence, differences in ash weight were very low since bone growth and mineralisation was at its maximum. Significant differences between treatments were detected for precaecal P digestibility in this study, but not for pcdP or ash weight. That means that more P was absorbed, but not stored in bones, probably leading to a higher P excretion with urine which was not analysed here (Hurwitz et al., 1978; Rodehutscord et al., 2012). An excessive P intake can even have negative effects on bone development (Vorland et al., 2017). By a high dietary P supply, especially in combination with low dietary Ca, the bone matrix protein osteopontin and the parathyroid hormone are increased. Both were identified to increase bone resorption. This may explain the numerically marginal decreased tibia ash weight with increasing phytase supplementation in the study described in Manuscript 2. Both tibia and foot ash were only analysed in Manuscripts 1 and 3. In Manuscript 3, Japanese quail were used instead of broiler chickens. At the end of the experiment, these quails were only 15 d

old, which is relatively young for foot ash analyses. In contrast to Manuscript 1 and the animals from Manuscript 4 described above, tibia ash weight (45.8 mg) was higher than foot ash weight (44.8 mg; $p < 0.001$) in this study. Nonetheless, tibia and foot provided very similar correlation coefficients with the different quantitative P measurements. The absolute amount of foot ash provided higher correlation coefficients than the tibia for pcdP and precaecal P digestibility. For the ash concentration data, the tibia showed higher values. Correlation coefficients measured in quail were generally lower than in the broiler experiments, which could be related to the study design that did not involve variation in P supply of birds. Digestibility values in this trial were determined by quantitative determination of P intake and P in excreta. Quail were not colostomised and digesta was not analysed. Since the P supply was below the requirement, P concentration in the urine can be assumed to be very low (Rodehutscord et al., 2012). Therefore, P digestibility can be calculated from P intake and P excretion in faeces, but not precaecal P digestibility. The genetic correlations between P digestibility or P retention and ash data calculated in Manuscript 3 were also minimally higher for foot than for tibia ash, indicating that foot ash is also a good trait for genetic analyses. Correlation coefficients obtained for data used in Manuscript 1 are higher, but with a similar predication. Tibia ash shows marginally lower correlation coefficients with quantitative P measurement traits than foot ash. The precaecal P digestibility values were most likely not significant for this study because there were four treatments with graded DCP supplementation. The precaecal P digestibility decreased with increasing DCP supplementation, while bone ash weight increased. With graded phytase supplementation, both traits increased. In the study resulting in Manuscript 4, the tibia was only taken from some birds to compare tibia and foot regarding the mineral concentrations in ash. The previous studies already showed the suitability of foot ash as an alternative for tibia ash to detect the relative bioavailability of P. The very high correlation coefficients obtained for foot ash with pcdP and rP confirmed this.

To conclude, in the current state of knowledge it is not possible to decide whether tibia or foot ash better reflects relative bioavailability of P. Both bone fractions provided very similar results in almost all studies but have distinct characteristics during processing. No preference for one of the bone fractions could be deduced. It can be recommended to use tibia or foot ash, depending on the specific situation.

Table 3: Pearson correlation coefficients between precaecally digestible P, precaecal P digestibility or retained P and the absolute amount (mg) or concentration (% of DM) of tibia or foot ash weight observed for data used in Manuscripts 1-4

	Precaecally digestible P (g/kg DM)	Precaecal P digestibility (%)	Retained P (g/animal/d)
Manuscript 1 Tibia mg	0.64	n.s.	0.92
Manuscript 1 Tibia %	0.69	n.s.	0.90
Manuscript 1 Foot mg	0.68	n.s.	0.94
Manuscript 1 Foot %	0.70	n.s.	0.83
Manuscript 2 Tibia mg	n.s.	n.s.	0.38
Manuscript 2 Tibia %	n.s.	n.s.	n.s.
Manuscript 3 Tibia mg[1]	0.52	0.53	0.76
Manuscript 3 Tibia %[1]	0.35	0.36	0.37
Manuscript 3 Foot mg[1]	0.54	0.54	0.75
Manuscript 3 Foot %[1]	0.27	0.28	0.29
Manuscript 4 Foot mg	0.91	0.49	0.94
Manuscript 4 Foot %	0.92	0.43	0.91

n.s. = not significant ($p > 0.05$)

[1] In Manuscript 3 the values were calculated from P intake and excretion, digesta was not analysed; therefore, total tract P digestibility and digestible P are reported for this study

Manuscript 1: 792 unsexed broiler chickens allocated to 11 treatments in 66 pens (n = 66); animals were fed either a P/Ca reduced basal diet, or the same diet supplemented with graded supplementation levels of a mineral P source or one of two phytase products

Manuscript 2: 440 unsexed broiler chickens allocated to 5 treatments in 40 pens (n = 40); animals were fed a control diet adequate in all nutrients, three diets with graded supplementation levels of phytase or one diet supplemented with *myo*-inositol

Manuscript 3: Each of the 887 unsexed Japanese quail received the same low P diet (n = 887)

Manuscript 4: 630 male broiler chickens allocated to nine treatments in 63 pens (n = 63); animals were fed a low P diet with or without mineral P, phytase, and coccidiostat supplementation

3.2 Comparison of body sides

When bones are chosen for P evaluation, not only the bone itself could be of importance, but also its location in the body. The most frequently used bones for the purpose of P evaluation are located in the leg. Because they exist in duplicate, one side must be chosen if only one bone per animal is to be analysed. Some methodological investigations were done using both sides to analyse different methods on the same animal. For example, Garcia and Dale (2006) used the tibia and foot from both legs to compare the effect of fat extraction. While the left tibiae and feet were fat extracted,

the right ones did not receive this treatment. For such investigations, both sides must provide identical results when treated uniformly. Even if only one side of each animal is used, it is important to know if there is a difference between the two sides. Fleming et al. (1999) described significant differences in humeral breaking strength between left and right limbs of laying hens of different strain and age. A substantial variation between mechanical bone traits such as bone breaking strength or torsional stiffness for both sides of long bones of rabbits was also reported by White et al. (1974). Nevertheless, no significant differences between the left and right bones were detected in this study. They concluded that a biologically normal variation exists, which is smaller between the left and the right side of one animal than between two animals. Accordingly, fewer animals are necessary when using both limbs to compare two methodological aspects to achieve a given level of statistical significance. Differences in bone mineralisation between the left and the right body side of poultry have not been analysed yet.

Bone measurements such as bone weight or length from two limbs are used as an animal welfare indicator for broiler chickens. The assumption is that both body sides develop symmetrically since they must cope with the same environmental conditions. It is assumed that asymmetrical development is either caused by genetic factors or reflects development instability, caused by challenging rearing conditions (Møller and Swaddle, 1997; van Nuffel et al., 2007). Hence, differences between body sides may indicate a reduced fitness and welfare of an animal. Another explanation for morphological asymmetry is a one-sided dominance due to a preferred limb. This one-sided dominance does not only influence muscles, but also bones and is known in humans, rats, rabbits and frogs (Chhibber and Singh, 1970; Singh, 1971; Kimura et al., 1975; Fox et al., 1995). It seems possible that it also exists in poultry.

Therefore, additional measurements were made adjunct to the study described in Manuscript 4. The DM and ash weight of the left and the right foot from 70 male broiler chickens allocated to seven pens were detected. The birds belonged to the same treatment and received a P/Ca reduced diet with phytase and coccidiostat supplementation. Performance data of these animals were on a similar level as the performance objectives of the breeding company, indicating that the rearing conditions were adequate (Aviagen, 2012). A one-way ANOVA using the body side as a fixed effect and the animal as a random effect showed a significant effect of body side for the foot ash amount ($p = 0.044$; Table 4). Consequently, when all feet are used from the same side, the left feet provide other results than the right ones. However, although the body side effect on foot ash was significant, the difference between left and right was small. When the ash weight is expressed as the concentration of the foot DM weight, no significant difference between the left and the right foot was detected ($p = 0.160$).

Table 4: Differences between the left and the right foot of male 24/25-day-old broiler chickens[1] detected for foot ash amount (mg) and concentration (%)

	left foot	**right foot**	***pooled SEM*[2]**	***p*-value**
Foot ash (mg)	1079	1074	*14.9*	0.044
Foot ash (%)	13.1	13.1	*0.089*	0.160

[1] All birds (n = 70) were part of the study described in Manuscript 4 and received a P/Ca reduced diet with phytase and coccidiostat supplementation

[2] SEM = standard error of the mean

The feet used in this study were cut at the *articulatio intertarsalis*, so the tarsometatarsus and all toe bones were analysed together with the skin, surrounding tissues and claws. The ash is assumed to consist mainly of minerals stored in the bone while the DM is more influenced by the surrounding soft tissue. Skin or ligaments are more difficult to standardise in sampling than well-defined bones. Therefore, the DM weight and consequently also the ash concentration, are more susceptible to sampling inaccuracy. Hence the non-significant side effect on ash concentration in contrast to the significant effect on ash weight could either be interpreted as an effect on bone mineralisation, compensated by the other tissues, or it was related to potential sampling inaccuracy.

The comparison between the left and right side provides no prediction about the difference between the two feet independent from the body side and the consequences for sampling. No significant difference between left and right for ash concentration could either mean the left and right foot provide the same results or the distribution of animals with the heavier foot on the left or the right side was well-balanced in this study. Therefore, the feet of each animal were sorted as lighter and heavier instead of left and right. A total of 40 animals (57 %) showed their heavier foot ash weight on the left side while it was 45 animals (64 %) when using the ash concentration. One animal had identical weights for both sides of ash weight. A significant difference between the heavier and lighter side was analysed for both traits ($p < 0.001$, respectively; Table 5). However, the difference between the two sides was small (15 mg for foot ash weight and 0.21 % for foot ash concentration), which makes the relevance appear to be minor.

In conclusion, the two feet of one animal can differ in ash weight and concentration despite adequate rearing conditions, indicating a normal physiological process caused by heredity or one-sided dominance. Although there was a tendency for the left foot to be heavier, there was no clear effect of one side. Therefore, using both legs of the same animal may be appropriate for methodological investigations when the body side is considered as a random effect. The included manuscripts of the present thesis used foot or tibia ash traits determined from the right side of each animal, respectively. After analysing the data described in this chapter, a better procedure

would be to randomise the sampling side of each animal. Another, more laborious method was described by Scholey and Burton (2017). They analysed bones from both legs and used the mean from both sides of each animal. Since the analysis described herein was only done with foot ash, it could be useful to do it again with more animals and different bone fractions.

Table 5: Differences between the heavier and the lighter foot of male 24/25-day-old broiler chickens[1] detected for foot ash amount (mg) and concentration (%)

	lighter foot	**heavier foot**	***pooled SEM*[2]**	***p*-value**
Foot ash (mg)	1067	1082	*21.7*	<0.001
Foot ash (%)	13.0	13.2	*0.088*	<0.001

[1] All birds (n = 70) were part of the study described in Manuscript 4 and received a P/Ca reduced diet with phytase and coccidiostat supplementation

[2] SEM = standard error of the mean

3.3 Comparison of ash amount and concentration

There are two common ways to present ash values: the absolute amount of ash contained in a bone or foot (g or mg), and the ash concentration in a bone fraction (% of DM or g/kg). Most often, the ash concentration is used in literature. The ash concentration is calculated from two absolute values, the ash amount is related to the DM weight of the respective bone fraction. Therefore, it does not reflect the bone weight which is highly related to the bone size. The absolute ash amount value shows the total amount of minerals contained in the bone. This amount is affected by the weight and consequently the size of a bone and its mineralisation, as already explained by Li et al. (2015) and Linde (2018). The bone size and mineralisation vary between animals and are affected by factors such as age, breed, feed intake, and phytase supplementation (Huyghebaert et al., 1980; Hall et al., 2003). In contrast, very similar ash concentrations can be detected in bones varying widely in weight and size (Li et al., 2015).

Several authors compared the suitability of tibia ash amount and concentration for the purpose of P evaluation with different approaches of regression analysis. In all studies, ash data were either plotted against non-phytate P or total P supply. Shastak et al. (2012a) reported a much higher R^2 and lower standard errors for the absolute ash amount compared to ash concentration. They did not only use the tibia of broiler chickens as evaluation trait, but also tarsometatarsus and toe, which behaved very similar. In the study of Li et al. (2015), the R^2 was very similar for both traits (ash amount: 0.81, ash concentration: 0.84). The absolute amount of tibia ash was more suitable than the ash concentration in regard to determining the relative bioavailability of different P sources (Coon et al., 2007) and the detection of phytase efficacy (Li et al., 2015). Huyghebaert et al. (1980)

found a very similar behaviour of regressions between the absolute amount of tibia ash or P retention and the intake of non-phytic P. The regression lines of both traits were nearly parallel when comparing two different experiments. The regression lines of tibia ash concentration, in contrast, showed different behaviour. For this trait, the same ash concentration values were detected for different P intake levels. This was not the case for ash amount or P retention. Shastak et al. (2012a) compared different response criteria by calculating the ratios of slopes for different mineral P sources. The slope ratios of ash data were overall not very close to the one of P retention in this study, with ash concentration being closer at an animal age of 21 d than ash amount. When birds were 35 d old, the absolute ash amount traits ranked closer to P retention than the concentration. Consequently, the animal age may also affect the suitability of ash data in biological availability studies. However, all authors concluded the absolute amount of tibia ash is the more sensitive trait for P evaluation compared to ash concentration.

Similar comparisons between ash amount and concentration were not made before for foot ash. In contrast to the tibia, the complete foot contains soft tissues surrounding the bones and therefore more soft tissues. Hall et al. (2003) account the organic bone components left after water and lipid removal being responsible for ash concentration to be less sensitive. All authors who investigated the suitability of foot ash as P evaluation trait used ash concentration values. They obtained a high relationship between foot and tibia ash concentration values (Table 1). These results suggest that the ash contained in the adhering soft tissues has no negative effects on the suitability of the ash concentration. Garcia and Dale (2006) also showed a high relationship between dietary available P and foot ash concentration ($R^2 = 0.90$), indicating that the concentration of foot ash is a suitable P evaluation trait.

Correlation coefficients presented in Table 3 can be used to compare ash amount and concentration values obtained in the own studies. As already described in Chapter 3.1.3, rP is the most suitable trait to investigate the relationship of ash data with a quantitative P measurement trait. The high P supply in all treatments of the study used in Manuscript 2 prevented high correlation coefficients in this respect because the variation in the response traits was apparently too small. It is notable, however, that a significant correlation coefficient between the absolute tibia ash amount and rP was calculated ($R = 0.38$) while the one between the ash concentration and rP was not significant. The other studies conducted with broiler chickens, described in Manuscript 1 and 4, included treatments with more variation in P supply. Both resulted in very similar correlation coefficients for ash amount and concentration with rP, with mostly only marginally higher values for the absolute ash amount. For the study resulting in Manuscript 4, only foot ash data were used. For this study, the difference between the correlation coefficients of ash amount ($R = 0.94$) and

concentration (R = 0.91) with rP was very small, indicating that the higher amount of ash contained in the adhering soft tissues included in the foot ash concentration seemed not to be of importance here. For the data used in Manuscript 1, the difference between ash amount (R = 0.92) and concentration (R = 0.90) was very similar for the relationship between tibia ash and rP. For foot ash, this difference was higher (ash amount: R = 0.94, ash concentration: R = 0.83). The reason for this remains unclear. Probably it is related to the used treatments. Three different supplements, two phytase products and DCP, were compared in the study described in Manuscript 1. Because they were fed in graded supplementation levels, regressions could be calculated between phytase supplementation or g P from DCP on the x-axis and different P evaluation traits on the y-axis. The slopes ratios between two supplements were used for efficacy calculations. When comparing the slope ratios of different traits, it depends on the used supplements whether ash amount or concentration showed slope ratios closer to rP.

When examining the quail data used for Manuscript 3, differences between ash amount and concentration were much bigger. The correlation coefficients between absolute ash amount data and rP were higher for both tibia (ash amount: R = 0.76, ash concentration: R = 0.37) and foot (ash amount: R = 0.75, ash concentration: R = 0.29) than between both bone fractions and ash concentration. It seems unlikely, that the poultry species accounts for the higher difference between ash amount and concentration than detected in the broiler studies. The subjective perception when preparing feet for drying was that the soft tissues of quail were smaller than those of broilers. A possible explanation is the P supply of the animals and the treatments examined in the respective studies. All quail received the same P reduced diet while broilers in the studies used for Manuscripts 1 and 4 were distributed in various treatments with very different P supply. Probably the P supply of quail was even scarcer than for broilers fed with P reduced diets. Therefore, the variation between animals was smaller for quail than for broiler in the conducted experiments, which was better reflected by the absolute ash amount. This may confirm the assumption that absolute ash amount is the more sensitive P evaluation trait compared to ash concentration.

To summarise, observations indicate that the absolute amount of ash is at least as suitable as ash concentration for P evaluation. Presumably, it is the even more accurate trait. Additionally, the absolute ash amount is the easier to obtain trait because it can be directly measured without any calculations and the determination of DM content in the respective bone fraction is not necessary.

3.4 Selection of birds

The analysis of tibia or foot ash is easier to perform than other analyses such as digesta analyses, but also laborious and time-consuming. Capacity in the laboratory is mostly limited. Therefore, it often comes to the question of how many birds should be used for ash analysis and how birds should be chosen from a group to obtain representative results. One principle in statistics is that a random sample should represent the whole population. Consequently, a random animal should represent the entire pen. However, this principle is only valid for large samples (Cohen, 1991) and the sample size in animal experiments is often small. Different approaches of the sampling method of animals for bone ash data can be found in the literature: while some authors used all animals for analyses (Broz et al., 1994; Banks et al., 2004), others selected specified animals. Waldroup et al. (2000) used two birds representing the mean BW out of five in the pen, Shaw et al. (2011) selected three random animals out of eight and Viveros et al. (2002) two random animals out of eight. Walk et al. (2014) decided to analyse four birds representing the mean BW out of twenty, and Adeola and Walk (2013) examined the four heaviest out of eight birds of each pen. Smith and Kabaija (1985) used three (out of ten) and Yan et al. (2001) two (out of six) not closer defined birds per pen. However, the effect of the selection method on the reliability of ash data as P evaluation trait has not been studied. Based on the experiments contained in this thesis, was simulated how different selection procedures may influence results. The hypothesis was that the bird number and selection method affect the results of bone ash data.

3.4.1 Selection of individual birds

The most feasible procedure to select single animals from a group is to weigh all the birds after slaughter and sort them by their BW. One or more animals can either be chosen by the rank position of their BW or by a random number. The broiler chickens used for the study described in Manuscript 1 were sorted by their BW, whereby 1 was the lightest and 12 the heaviest animal in one pen. Birds number 2, 5, 8, and 11 were chosen for analyses of foot and tibia ash. In case that not all animals of one pen survived the experimental period, an appropriate schema was chosen. This selection method should ensure to use animals of different BW categories to represent the whole spectrum of animals. The heaviest and lightest bird were not used as they are assumed to be possible outliers with an extremely high or low BW, which is supposed to influence bone ash weight. The four animals per pen resulted in a total of 288 birds for bone ash analyses. To simulate what could happen if only one defined animal per pen was selected, the four animals were distributed to four BW categories: A (bird number 2), B (bird number 5), C (bird number 8), and D (bird number 11). Three different supplements, DCP and two different phytase products (Natuphos® E 5000 G (NE) and Natuphos® 5000 G (N)), were used in graded supplementation

levels in addition to a low P basal diet in this study. Ash data were used to calculate linear regressions with the phytase supplementation or g P from DCP on the x-axis and ash amount or concentration on the y-axis (Figure 1).

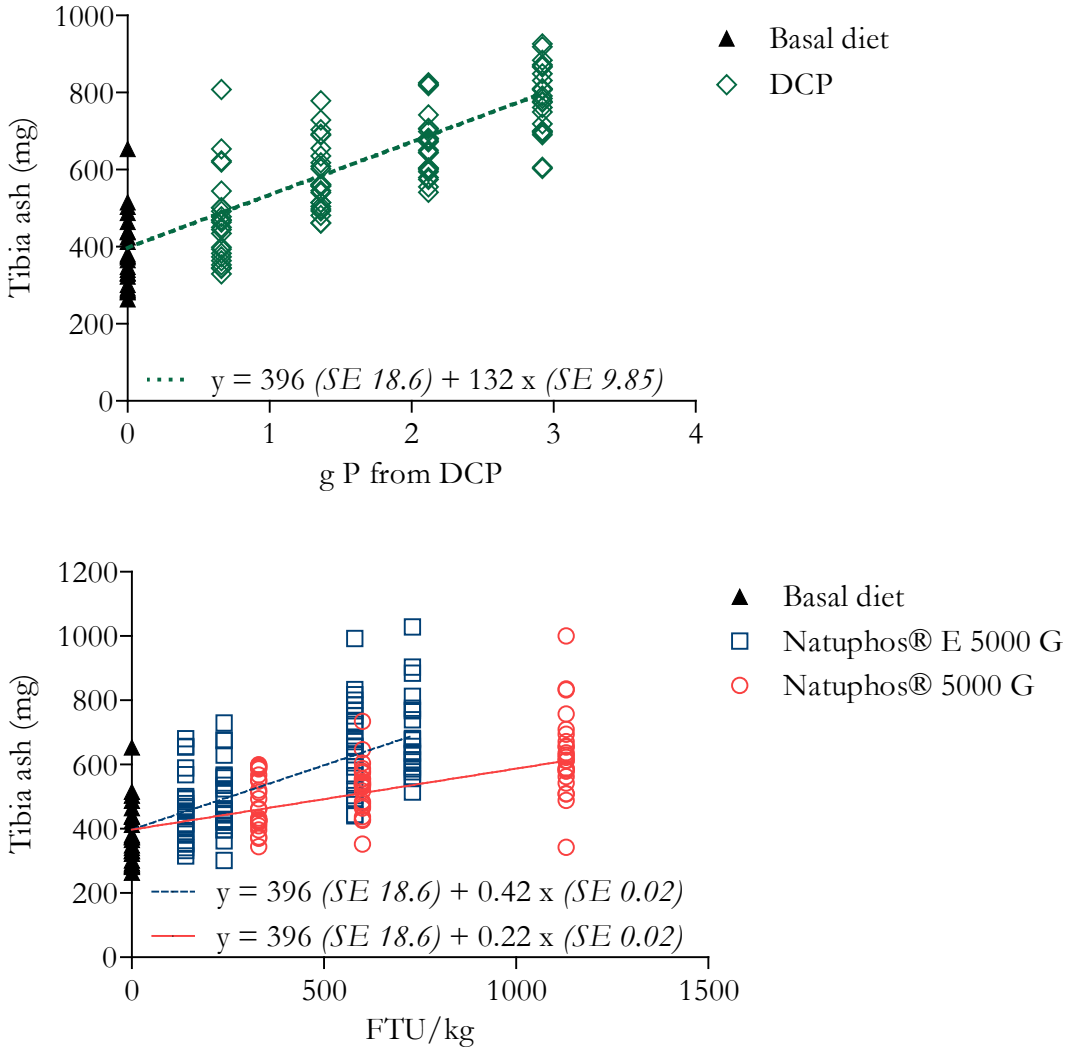

Figure 1: Effect of supplementation of dicalcium phosphate (at the top) or the phytase products Natuphos® E 5000 G and Natuphos® 5000 G (at the bottom) on tibia ash amount using birds from all body weight categories

All birds were part of the study described in Manuscript 1 and received a low-P diet without (basal diet) or with graded supplementation levels of dicalcium phosphate (DCP; 0.7, 1.4, 2.1, and 2.9 g P/kg from DCP) or one of the phytase products Natuphos® E 5000 G (125, 250, 500, and 750 FTU/kg) and Natuphos® 5000 G (250, 500, and 1000 FTU/kg). Each symbol denotes for one animal (n = 24 birds per treatment). SE = standard error

Regressions for the three supplements were calculated simultaneously using the MIXED procedure of SAS with the basal diet as common intercept for DCP, NE, and N. Linearity was tested using diet-specific means as lack of fit (α = 0.05). The following model was chosen:

$$y_{ijk} = \mu w_k + \beta_{NE} c_{NE_{ij}} + \beta_N c_{N_{ij}} + \beta_{DCP} c_{DCP_{ij}} + w_k \beta_{NE} c_{NE_{ij}} + w_k \beta_N c_{N_{ij}} + w_k \beta_{DCP} c_{DCP} + b_j + e_{ijk}$$

where μw_k represents the common intercept of the k^{th} BW category, β_{NE}, β_N, and β_{DCP} represent the three slopes for supplement NE, N, and DCP, respectively. $c_{NE_{ij}}$, $c_{N_{ij}}$, and $c_{DCP_{ij}}$ are the concentrations of each supplement in the pen with the i^{th} treatment and the j^{th} localisation within the barn. The localisation effect within the barn b_j (block) was taken as random effect and e_{ijk} is the error of y_{ijk} from the k^{th} BW category within the ij^{th} pen. In case of non-significant interactions (α = 0.05) between the BW category w_k and the slope β of one supplement, the interaction was removed and a common slope for all BW categories estimated. These calculations were done for all four birds selected for ash analyses together and for the four BW categories individually. Significant differences between slopes were detected using t-tests.

For all traits and both slope ratios (DCP/NE and DCP/N), BW category D provided the lowest slope ratio and B the highest. The slope ratios indicate the amount of the respective phytase product that is needed to cause the same increase in ash data as 1 g P from DCP. Consequently, when using only birds from BW category D, less phytase would be necessary to replace 1 g P from DCP than when using birds from BW category B. The slope of phytase N was lower than for NE. This means a higher amount of N is needed to replace 1 g P from DCP than from NE. While the slopes for DCP varied between BW categories, slopes for NE and N were the same for all BW categories. Therefore, the maximum difference between two BW categories is higher for the slope ratios of DCP/N (slope ratio B – slope ratio D = 171 (foot ash amount) to 369 (foot ash concentration)) than for the one of DCP/NE (slope ratio B – slope ratio D = 95 (foot ash amount) to 200 (foot ash concentration)). Hence, the relevance of the choice of selection is different and depending on the supplement under investigation. The ranking of BW categories was the same for the slopes of all analysed traits (D < C < all < A < B). However, both tibia and foot ash amount had smaller differences between the highest and the lowest BW category than the ash concentration of tibia and foot. Therefore, the selection method has a greater influence on phytase efficacy estimate when using ash concentration as evaluation trait compared to ash amount. Assuming that all four birds together provided the most reliable results since the highest number of animals was used, the highest and lowest deviation from this category varied between traits, but not between the two ratios of one trait. For tibia ash amount and foot ash concentration, slope ratios from BW category A had the lowest deviation from all animals and B the highest. For tibia ash concentration

and foot ash amount, slope ratios of animals from BW category C differed the least from all animals and D most. In summary, these results confirm that the way of animal selection affects the results of product efficacy estimated with ash data when only one defined animal is chosen from a group. However, it is not possible to recommend a selection method.

Table 6: Analysis of variance (ANOVA) of the effects used for the regressions between tibia or foot ash amount (mg) or concentration (%) and the level of three supplements[1] analysed for different body weight categories[2] and the slopes estimated for these regressions

	Tibia ash				Foot ash			
Slopes	**mg**	***SE*[3]**	**%**	***SE*[3]**	**mg**	***SE*[3]**	**%**	***SE*[3]**
N[4]	0.22	*0.02*	0.0055	*0.0006*	0.24	*0.014*	0.0019	*0.0002*
NE[4]	0.42	*0.02*	0.011	*0.0008*	0.43	*0.019*	0.0035	*0.0003*
DCP A	135[abc]	*9.85*	4.33[a]	*0.35*	142[ab]	*8.61*	1.44[ab]	*0.13*
DCP B	157[a]	*9.85*	4.40[a]	*0.35*	151[a]	*8.61*	1.63[a]	*0.13*
DCP C	119[bc]	*9.85*	3.07[bc]	*0.35*	126[bc]	*8.61*	0.96[c]	*0.13*
DCP D	115[c]	*9.85*	2.71[c]	*0.35*	110[c]	*8.61*	0.93[c]	*0.13*
DCP all	132[b]	*9.85*	3.63[b]	*0.35*	132[b]	*8.61*	1.24[b]	*0.13*
ANOVA								
BW	<0.001		<0.001		<0.001		<0.001	
NE	<0.001		<0.001		<0.001		<0.001	
N	<0.001		<0.001		<0.001		<0.001	
DCP	<0.001		<0.001		<0.001		<0.001	
BW × N	0.290		0.062		0.572		0.409	
BW × NE	0.153		0.773		0.412		0.634	
BW × DCP	0.027		<0.001		0.024		<0.001	

[1] All birds (n = 24 animals per treatment) were part of the study described in Manuscript 1 and received a low-P diet without (basal diet) or with graded supplementation levels of dicalcium phosphate (DCP; 0.7, 1.4, 2.1, and 2.9 g P/kg from DCP) or one of the phytase products Natuphos® E 5000 G (NE; 250, 500, and 750 FTU/kg) and Natuphos® 5000 G (N; 250, 500, and 1000 FTU/kg)

[2] The 12 birds of each pen were sorted by increasing body weight (BW) and defined birds selected to represent different BW categories: A = bird 2, B = bird 5, C = bird 8, D = bird 11, all = A + B + C + D

[3] SE = standard error

[4] The non-significant interactions between BW and N or NE were removed from the model and a common slope of all BW categories was calculated for these supplements

[a–c] Means within a column not showing a common superscript are significantly different ($\alpha = 0.05$)

Table 7: Slope ratios of regressions between tibia or foot ash amount (mg) or concentration (%) and phytase supplementation (Natuphos® E 5000 G (NE) or Natuphos® 5000 G (N)) or supplementation of dicalcium phosphate (DCP) for different body weight categories of broiler chickens[1]

	Body weight category[2]				
Slope ratios	**A**	**B**	**C**	**D**	**All (A+B+C+D)**
Tibia ash mg					
DCP/N	614	714	541	523	600
DCP/NE	321	374	283	274	314
Tibia ash %					
DCP/N	787	800	558	493	660
DCP/NE	394	400	279	246	330
Foot ash mg					
DCP/N	592	629	525	458	550
DCP/NE	330	351	293	256	307
Foot ash %					
DCP/N	758	858	505	489	653
DCP/NE	411	466	274	266	354

[1] All birds (n = 24 animals per treatment) were part of the study described in Manuscript 1 and received a low-P diet without (basal diet) or with graded supplementation levels of dicalcium phosphate (DCP; 0.7, 1.4, 2.1, and 2.9 g P/kg from DCP) or one of the phytase products Natuphos® E 5000 G (250, 500, and 750 FTU/kg) and Natuphos® 5000 G (250, 500, and 1000 FTU/kg). Slopes used for the calculations are shown in Table 6

[2] The 12 birds of each pen were sorted by increasing body weight and defined birds selected to represent different BW categories: A = bird 2, B = bird 5, C = bird 8, D = bird 11

In the study described in Manuscript 2, tibiae of all birds were used for ash analyses. However, the variation of P supply was very low and resulted in no significant differences in ash data between treatments. Therefore, further investigations regarding the selection of birds are not possible for this study.

3.4.2 Comparison of defined and randomly selected birds

Foot ash data of each of the ten animals per pen were used for the study described in Manuscript 4 to analyse relative bioavailability of P. These data were also used for further analyses to simulate the use of defined animals for the evaluation of ash data. The 560 birds were allocated to eight experimental treatments designed as a 2 × 2 × 2-factorial arrangement. They were fed diets without (P/Ca-) or with (P/Ca+) monocalcium phosphate, without (Phy-) or with (Phy+) phytase supplementation, and without (Coc-) or with (Coc+) coccidiostat supplementation. The additional treatment described in Manuscript 4 providing coccidiostat supplementation in the starter, but not in the grower diet was not used for the following analysis. Birds were weighed after slaughter and sorted by increasing BW. A three-way ANOVA with the P/Ca, phytase and coccidiostat levels as

fixed effects and the block and pen as random effects was performed for different numbers of animals chosen in specific ways:

- All ten birds of each pen
- Birds of each pen were divided into 3 BW categories (light: birds 1-3, middle: birds 4-7, heavy: birds 8-10) and one bird per BW category randomly selected
- 2 birds per pen with a mean BW (birds 5+6)
- 4 randomly selected birds/pen
- 3 randomly selected birds/pen
- 2 randomly selected birds/pen
- 1 randomly selected bird/pen

For the random selection, the respective quantity of random numbers between 1 and 10 was created by the PROC PLAN procedure of SAS. Random numbers are used for animal selection to avoid the unconscious preference of specific animals when picking random animals directly after slaughter. When only one animal was selected, the pen was not used as a random effect in the statistical analysis. Heterogeneity of error variances between treatments was tested for each selection method, and the model with the smallest Akaike information criterion was used. Results of the ANOVA for foot ash amount (Table 8) and concentration (Table 9) showed distinctly increased pooled standard errors of the mean when fewer animals were used for the analysis. Consequently, the estimates are more inaccurate and the likelihood to detect significant differences is lower.

The three-way interaction between P/Ca level, phytase and coccidiostat supplementation was only significant for all animals and the 2 mean animals when examining the ash amount data, pointing towards the most sensitive analysis within these selection methods. For the ash concentration, all birds, 3 BW categories, 4 random birds and 1 random bird resulted in a significant three-way interaction. However, different selection methods provided different significant differences between treatments. The ranking of foot ash amount or concentration between treatments also varied mostly with different selection methods. Only treatments P/Ca+Phy-Coc- (third highest ash amount and concentration), P/Ca-Phy-Coc+ (second lowest ash amount and concentration), and P/Ca-Phy-Coc- (lowest ash amount and concentration) had the same ranking for all selection methods. This means different results would be obtained with different selection methods. Only the variant of using 2 mean birds had the same ranking of treatments as all birds for ash amount.

When observing the ash concentration, both 4 and 2 randomly selected birds showed the same ranking of treatments as all animals.

Table 8: Effect of number and selection method[1] of birds on the evaluation of foot ash amount of 25-day-old broiler chickens fed different experimental diets[2]

	All (10 birds)	3 BW categories	2 birds mean BW	4 random birds	3 random birds	2 random birds	1 random bird
Estimated treatment means							
P/Ca-Phy-Coc-	568[e]	556	579[d]	565	554	581	592
P/Ca-Phy-Coc+	638[d]	651	663[d]	636	607	633	660
P/Ca-Phy+Coc-	1079[c]	1103	1125[bc]	1098	1093	1108	1162
P/Ca-Phy+Coc+	1074[c]	1076	1051[c]	1075	1079	1125	1051
P/Ca+Phy-Coc-	1145[b]	1119	1167[b]	1141	1194	1159	1178
P/Ca+Phy-Coc+	1077[c]	1084	1095[bc]	1099	1091	1091	1030
P/Ca+Phy+Coc-	1277[a]	1282	1263[a]	1306	1304	1265	1401
P/Ca+Phy+Coc+	1281[a]	1264	1276[a]	1275	1282	1334	1268
pooled SEM[3]	*22.6*	*32.7*	*34.5*	*29.7*	*34.5*	*47.2*	*57.3*
ANOVA							
P/Ca	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Coccidiostat	0.968	0.856	0.589	0.747	0.361	0.589	0.048
P/Ca×Phy	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
P/Ca×Coc	0.019	0.161	0.439	0.131	0.086	0.600	0.140
Phy×Coc	0.942	0.217	0.413	0.288	0.881	0.435	0.305
P/Ca×Phy×Coc	0.008	0.106	0.010	0.186	0.123	0.184	0.228

[1] Data basis for the three-way ANOVA were either all (10) birds of one pen (n = 560) or some specified selected: 3 body weight (BW) categories = animals were divided into 3 BW categories (light, mean, heavy) and one random bird of each BW category was selected; 2 animals mean BW = birds were sorted by increasing BW and birds number 5 and 6 were used for the analysis

[2] All birds were part of the study described in Manuscript 4 and received a diet without (P/Ca-) or with (P/Ca+) monocalcium phosphate, without (Phy-) or with (Phy+) phytase and without (Coc-) or with (Coc+) coccidiostat supplementation

[3] SEM = standard error of the mean

[a–e] Means within a column not showing a common superscript are significantly different ($\alpha = 0.05$)

These results indicate that the number of animals which should be used for ash analyses depends on the expected differences between treatments. Still, the best practice is to use all animals of one pen. The variant of using 2 mean animals provided the closest results for ash amount regarding significances and ranking of treatments. The absolute ash amount better reflects the bone size and therefore also the animal weight than ash concentration. Accordingly, animals with mean BW reflect better ash amount than ash concentration. The use of 3 BW categories was simulated to make a compromise between representing the whole spectrum of different BW in the pen and

using a random sample. When using three completely random samples, it is also possible that the three lightest animals are chosen. For foot ash amount, results of these two selection methods were very similar, implying that the use of defined animals is not superior to random animals for the here obtained data. For ash concentration, the three-way interaction was significant for 3 BW categories, but not for 3 randomly selected animals. Consequently, not only the expected difference between treatments is of importance for the decision on a selection method, but also the trait which should be analysed.

Table 9: Effect of number and selection method[1] of birds on the evaluation of foot ash concentration of 25-day-old broiler chickens fed different experimental diets[2]

	All (10 birds)	**3 BW categories**	**2 birds mean BW**	**4 random birds**	**3 random birds**	**2 random birds**	**1 random bird**
Estimated treatment means							
P/Ca-Phy-Coc-	9.46^{f}	9.25^{e}	9.75	9.54^{e}	9.26	9.45	9.49^{c}
P/Ca-Phy-Coc+	9.84^{e}	10.0^{d}	9.98	9.83^{e}	9.66	9.69	10.1^{c}
P/Ca-Phy+Coc-	13.3^{d}	13.3^{bc}	13.1	13.4^{c}	13.1	13.2	13.8^{b}
P/Ca-Phy+Coc+	13.1^{d}	13.1^{c}	13.3	12.9^{d}	13.1	13.0	13.3^{b}
P/Ca+Phy-Coc-	14.4^{b}	14.4^{a}	14.4	14.4^{b}	14.6	14.5	14.8^{a}
P/Ca+Phy-Coc+	13.8^{c}	13.7^{b}	14.0	13.7^{c}	13.9	13.8	14.1^{bc}
P/Ca+Phy+Coc-	14.9^{a}	14.6^{a}	15.1	14.8^{a}	15.2	14.8	14.7^{a}
P/Ca+Phy+Coc+	14.8^{a}	14.7^{a}	14.7	14.8^{ab}	15.0	14.5	14.8^{a}
pooled SEM[3]	*0.11*	*0.20*	*0.24*	*0.17*	*0.21*	*0.25*	*0.30*
ANOVA							
P/Ca	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Coccidiostat	0.117	0.931	0.516	0.070	0.329	0.217	0.636
P/Ca×Phy	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P/Ca×Coc	0.009	0.051	0.084	0.299	0.033	0.177	0.346
Phy×Coc	0.495	0.821	0.823	0.670	0.993	0.929	0.685
P/Ca×Phy×Coc	0.001	0.001	0.958	0.005	0.101	0.256	0.030

[1] Data basis for the three-way ANOVA were either all (10) birds of one pen (n = 560) or some specified selected: 3 body weight (BW) categories = animals were divided into 3 BW categories (light, mean, heavy) and one random bird of each BW category was selected; 2 animals mean BW = birds were sorted by increasing BW and birds number 5 and 6 were used for the analysis

[2] All birds were part of the study described in Manuscript 4 and received a diet without (P/Ca-) or with (P/Ca+) monocalcium phosphate, without (Phy-) or with (Phy+) phytase and without (Coc-) or with (Coc+) coccidiostat supplementation

[3] SEM = standard error of the mean

[a–f] Means within a column not showing a common superscript are significantly different ($\alpha = 0.05$)

To conclude, the number and selection method of animals for bone ash data can influence results. More experiments with bigger data sets are needed to elucidate if the use of multiple animals with mean BW is most suitable for ash amount when it is not possible to use all animals for ash analyses. In any case, it is necessary to describe the selection method of animals in publications to interpret the results adequately.

3.5 Bone ash data for phosphorus efficiency breeding

Besides the supplementation of phytase products, breeding for P efficiency is a possible tool to reduce the supplementation of mineral P to poultry diets. The breeding program can be related to the feed ingredients by increasing the amount of plant-derived phytase (Holme et al., 2012; Scholey et al., 2017) or by reducing the phytate in the crop (Raboy, 2020). In both options relating to the feed ingredients, the program´s success can be monitored with the help of an animal experiment where bone ash is analysed. Another possibility for P efficiency breeding is a program related to the animal (Diarra et al., 2010), where bone ash can be used as a proxy trait. The heritability estimated for bone ash data estimated in Manuscript 3 (0.230-0.342) was higher than that for PU (0.134). Also, strong genetic (0.462-0.549) and phenotypic (0.268-0.527) correlations were detected between foot and tibia ash data and PU. Consistent with other methodological considerations of this thesis, foot ash amount showed higher phenotypic and genetic correlations with PU than tibia ash amount or the ash concentration values of both bones. Additionally, the highest heritability among these bone ash traits was detected for foot ash amount. Consequently, foot ash amount seems to be the most suitable proxy trait for the purpose of breeding for PU.

Beck et al. (2016a) performed profound genetic analyses concerning PU including the detection of quantitative trait loci (QTL) (Beck et al., 2016b) with the same quails used for the experiment described in Manuscript 3. They considered PU a 'very-hard-to-measure trait' and investigated the complex biological relationship between PU, feed conversion, and BW gain. These relationships are essential to consider when the aim is to predict all the effects that a selection for high PU would have on the animals. It would be of interest to apply these structural equation models again to investigate the relationship between PU and bone ash data better. Bone ash showed higher genetic and phenotypic correlations with PU than the performance data and is easier to analyse than PU, especially under practical conditions. Thus, ash data might serve as proxy traits to improve PU in a breeding program. Genetic analyses of different bone traits have been reported several times in literature since bone health is an important issue in the poultry industry with a strong influence on animal welfare and economy of production. A high ash amount in the bone accounts for good mineralisation and therefore strong bones. Skeletal disorders are of high importance in the poultry industry and have multifactorial reasons. Housing conditions, genetics and nutrition, especially a

lack of minerals, are the most frequent causes for non-infectious leg disorders (Hafez and Hauck, 2005). González-Cerón et al. (2015) concluded bone quality traits like tibia ash concentration to have an additive genetic background which can therefore be improved through selection. Mignon-Grasteau et al. (2016) identified numerous tibia related QTL in their study using broiler chickens. However, to manage limited global rock phosphate reserves and to reduce eutrophication, it is vital to select not only on high bone ash amount but also on PU. Therefore, the selection of animals for breeding with bone ash as a proxy trait should be made with birds fed a P reduced diet to let the animals express their full genetic potential of PU. A P reduced diet was provided in the experiment described in Manuscript 3.

The underlying mechanism leading to the heritability estimation mentioned before needs to be elucidated in more depth, including the role of microbes. The gastrointestinal microbiota are involved in PU and might be influenced by breeding (Hufeldt et al., 2010; Schokker et al., 2015). Borda-Molina et al. (2020) analysed the microbiota composition of the quail used for Manuscript 3. They found differences in the abundance of some microorganisms between animals with low and high PU. Still, they could not differentiate if these differences were caused by differences in PU or vice versa. Other authors reported a relationship between microbiota composition and bone metabolism, inter alia by affecting the nutrient absorption (Hernandez et al., 2016; Pacifici, 2018). Significant correlations between microorganisms and bone ash data were also observed in the experiments described in Manuscript 1 and 4. Hence, microbial activity in the digestive tract may be one linkage between the genome of the animal and its P utilisation efficiency. Ponsuksili et al. (2020) also used samples from the quail described in Manuscript 3. They selected birds with high or low PU and did microRNA profiling of ileum tissue. They found functional pathways involved in phosphate or bone metabolism that were differentially expressed in high and low PU quails.

The relationship between all these processes needs to be examined more detailed for a targeted P efficiency breeding. Results obtained with Japanese quail need to be verified with target species such as broiler chickens and laying hens.

3.6 Conclusions and perspectives for future research

Investigations performed in this thesis showed the importance of a careful selection of methods when using bone ash data for the evaluation of relative P bioavailability. It would be helpful to have a standardised assay to obtain meaningful and more comparable estimates.

For the decision between tibia and foot ash, no clear preference of one bone fraction was determined. Using the tibia has the advantage of being a clearly defined bone, but it includes the risk of missing bone fragments during the laborious preparation process. The foot, in contrast, is very easy to handle because the soft tissues surrounding the bones do not have to be removed. However, standardisation of the segregation of soft tissues and skin is hardly possible. A strong relationship between tibia and foot ash was observed in literature and the conducted studies. Only marginal differences were detected between the P concentration in the ash of both bone fractions. The relationship with quantitative P measurements like retained P was also very similar between tibia and foot ash. An interesting aspect of further research would be the comparison of whole-body P concentration with the P concentration in both tibia and foot ash to obtain which bone fraction reflects the whole body best.

The evaluation of ash data from both body sides of broiler chickens showed significant differences between both ash amount and concentration of the heavier and lighter foot. This should be considered when planning to sample for bone ash data. A possibility is to randomise the body side of sampling for each animal. Since investigations for this methodological aspect were only done with foot ash, further studies using both foot and tibia ash from a higher number of animals could be helpful.

Several authors recommend using ash amount data instead of ash concentration. Observations described in this thesis confirm that the absolute amount of foot or tibia ash is at least as suitable as ash concentration for the evaluation of relative P bioavailability. The ash amount could be even more precise because the bone weight is included. An additional advantage is a direct measurement without any calculations and the determination of dry matter content, which are necessary for the investigation of ash concentration values.

A simulation of different approaches showed that the number of animals selected from a group for bone ash analyses and the way of selection affects the outcome of P evaluation studies. If possible, all animals involved in the experiment should be used for ash analyses. However, if a selection is inevitable, it should be made based on the expected differences between treatments, the intended response trait (ash amount or concentration), and the analysed supplements. To

further explore the influencing factors and to recommend a selection method, studies with bigger datasets are needed.

Japanese quail were used as model animals for broiler chickens in this thesis. However, direct comparisons were not included. An experiment using both poultry species simultaneously could help to elucidate species-specific effects. Different P levels in the diet should be used, and both bone ash data and precaecally digestible P should be analysed.

Bone ash data might play an important role in P efficiency breeding. Among the bone ash traits, foot ash amount showed the highest heritability and closest phenotypic and genetic correlations with P utilisation, indicating it might be most suitable for this purpose. Further investigations are needed to understand the complex relationship of all biological processes involved in P utilisation and to establish a successful breeding program.

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4 INCLUDED MANUSCRIPTS

4.1 Manuscript 1

RELATIVE PHYTASE EFFICACY VALUES AS AFFECTED BY RESPONSE TRAITS INCLUDING ILEAL MICROBIOTA COMPOSITION

Susanne Künzel[1], Daniel Borda-Molina[1], Tobias Zuber[1], Jens Hartung[2], Wolfgang Siegert[1], Dieter Feuerstein[3], Amélia Camarinha-Silva[1] and Markus Rodehutscord[1]

[1] *Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany*

[2] *Institute of Crop Science, University of Hohenheim, 70599 Stuttgart, Germany*

[3] *BASF SE, 68623 Lampertheim, Germany*

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Relative phytase efficacy values as affected by response traits, including ileal microbiota composition

Susanne Künzel,* Daniel Borda-Molina,* Tobias Zuber,* Jens Hartung,† Wolfgang Siegert,* Dieter Feuerstein,‡ Amélia Camarinha-Silva,* and Markus Rodehutscord*,[1]

*Institut für Nutztierwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany; †Institut für Kulturpflanzenwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany; and ‡BASF SE, 68623 Lampertheim, Germany

ABSTRACT The objective of this study was to compare the effects of graded inclusions of 2 phytase products and a mineral P source in broiler chickens using different response traits, including ileum microbiota composition. Eleven experimental diets were used. These were a low-P basal diet and diets supplemented with increasing levels of dicalcium phosphate (**DCP**), Natuphos E 5000 G (**NE**), or Natuphos 5000 G (**N**). The performance traits, prececal P digestibility, and tibia and foot ash results were subjected to regression analysis and slope ratios were used to compare the supplements based on the measured evaluation traits. In the microbiota analysis, total nucleic acids were extracted and the 16S rRNA gene was targeted for use in the amplicon sequencing process. Phylogenetic analysis was performed using Mothur, followed by a multivariate statistical analysis. The various response traits caused different estimates of relative efficacy. The mean results of all the response traits showed that a 1.75-fold increase in the activity of N was needed to achieve the same response as NE and the variability among the detected traits ranged from 1.59 (prececally digestible P intake) to 1.91 (amount of tibia ash). The mean slope ratio between DCP and NE was 311 and varied between 208 (ADG) and 349 (foot ash concentration). The mean slope ratio for phytase N with DCP was 552 and varied from 357 (ADG) to 640 (tibia ash concentration). The ileum microbiota composition was not different among the diets. A similar composition was driven in the abundance of *Lactobacillus crispatus, Lactobacillus salivarius,* and *Lactobacillus gallinarum.* The results suggest that different response traits cause markedly different estimates of relative phytase efficacy.

Key words: evaluation, method, digestibility, bone, microbiota

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INTRODUCTION

Different phytase products are used by the broiler industry. These phytases degrade *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) (**InsP$_6$**) and its salts (phytate), which are the major forms of P in plant feedstuffs. Phytate is only partially degraded by endogenous enzymes in the animal. Therefore, phytase supplements are currently being used to increase P utilization by the animal. Different phytase products have specific activity characteristics, such as an optimum pH or resistance to proteolysis, and thus may have different InsP$_6$ degradation efficiencies in the gastrointestinal tract. Depending on the genetic background, phytases start dephosphorylation of InsP$_6$ at a specific position on the inositol ring and follow a certain degradation cascade (Konietzny and Greiner, 2002). Two major groups are 6- and 3-phytases. The number indicates that dephosphorylation is initiated at the sixth and third phosphate group on the inositol ring, respectively.

There are various approaches currently used to determine available P in broilers. Prececally digestible P (**pcdP**) is the most established trait for quantitative purposes (WPSA, 2013), while bone-related data, BW gain, and blood inorganic P concentration are used to detect the relative P bioavailability (Shastak and Rodehutscord, 2013). The most often used bone for this kind of analysis is the tibia. However, other bones, such as the femur, toes, or the complete foot, are also described as response traits (Yan et al., 2005; Malloy et al., 2017). Shastak et al. (2012a) compared different bone measurements, pcdP, P retention, and blood inorganic phosphate in response to different mineral P supplements. They concluded that bone criteria and P retention or

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[1]Corresponding author: inst450@uni-hohenheim.de

pcdP led to different rankings for the values of the 2 mineral P sources tested. Hence, there may be differences in the relative values for different phytase products depending on the response trait that is measured.

To some extent, phytase is endogenously provided by the epithelial tissue of broilers and by some of the microorganisms in the gastrointestinal tract. *Lactobacillus* species, such as *L. salivarius, L. plantarum,* and *L. pentosus,* are able to produce enzymes with phytase-like activity (Lee et al., 2013; Sumengen et al., 2013; Amritha et al., 2017). Microorganisms in the gastrointestinal tract may contribute to the degradation of phytate and other fractions in the feed. The abundance of microbial groups can be influenced by feedstuffs and supplements, such as phytase or mineral P (Witzig et al., 2015; Borda-Molina et al., 2016). However, as far as can be ascertained, the effects of different phytase products on intestinal microbiota composition have not yet been compared.

The objective of this study was to compare 2 phytase products and dicalcium phosphate (**DCP**) using several response traits, including ileal microbiota composition. Each of the 3 supplements was fed at different inclusion levels, which meant that the results could be analyzed using regression analysis and slope ratios. The hypotheses were that the relative efficacy values of phytase products and DCP depend on the chosen response trait and that ileal microbiota composition responses differ among products.

MATERIALS AND METHODS

Birds and Housing

The trial was conducted at the Agricultural Research Station of the University of Hohenheim, Germany. All procedures were performed in accordance with the German Animal Welfare Legislation requirements and approved by the Regierungspräsidium Tübingen, Germany (project no. HOH 37/15 TE). A total of 792 unsexed Ross 308 broiler hatchlings were supplied by a commercial hatchery (Brüterei Süd GmbH & Co. KG, Regenstauf, Germany) and allocated to 66 pens (115 × 230 cm ground area, 260 cm height), which were stocked with 12 hatchlings each. On d 7, 6 pens were assigned to each of the 11 treatments according to a randomized complete block design. The birds were kept on wood shavings bedding until d 14, and on perforated floors from d 14 to d 22. Feed and tap water were provided for *ad libitum* consumption during the trial. The lighting program was 24 L:0 D until d 3, 22 L:2 D until d 7, 16 L:8 D until d 10, and 12 L:12 D until the end of the experiment. The temperature was set at 34°C on the day of placement and continuously decreased to 24°C on d 22. The well-being of the birds was checked at least twice daily.

Diets and Treatments

The birds received a commercial pelleted starter feed in the pre-experimental phase until d 7 (4150020 Club Kükenmaststarter, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). During the experimental phase (d 7 to 22), the birds were fed corn and solvent-extracted soybean meal-based pelleted diets (Table 1). The basal diet (**BD**) contained 4.5 g P/kg DM and 7.9 g Ca/kg DM but no mineral P or exogenous phytase. The other diets had a similar ingredient composition but contained increasing amounts of 1 of 3 supplements: DCP (0.7, 1.4, 2.1, and 2.9 g P/kg from DCP), NATUPHOS E 5000 G (**NE**; 250, 500, and 750 FTU/kg), or NATUPHOS 5000 G (**N**; 250, 500, and 1000 FTU/kg). The enzymes were supplied by BASF SE (Lampertheim, Germany). When DCP was added, the inclusion of calcium carbonate was adjusted to maintain

Table 1. Ingredient composition and analyzed concentrations of the experimental diets.[1]

Ingredient, g/kg	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000
Corn	575	575	575	575	575	575	575	575	575	575	575
Soybean meal	335	335	335	335	335	335	335	335	335	335	335
Rapeseed meal	30	30	30	30	30	30	30	30	30	30	30
Soybean oil	15	15	15	15	15	15	15	15	15	15	15
Calcium carbonate	14	14.5	15	15.5	16	14	14	14	14	14	14
Diamol[2]	15	11.3	7.5	3.8	-	15	15	15	15	15	15
Dicalcium phosphate	-	3.2	6.5	9.7	13	-	-	-	-	-	-
Vitamin/mineral mix[3]	5	5	5	5	5	5	5	5	5	5	5
TiO_2	5	5	5	5	5	5	5	5	5	5	5
Sodium bicarbonate	3	3	3	3	3	3	3	3	3	3	3
DL-methionine	2	2	2	2	2	2	2	2	2	2	2
Sodium chloride	1	1	1	1	1	1	1	1	1	1	1
Phytase (FTU/kg)	0	0	0	0	0	250	500	750	250	500	1000
Analyzed concentrations											
Ca (g/kg DM)	7.9	9.0	10.3	11.4	12.7	7.8	8.1	7.9	8.0	8.0	7.9
P (g/kg DM)	4.5	5.2	5.9	6.6	7.4	4.5	4.5	4.5	4.5	4.5	4.5
$InsP_6$-P (g/kg DM)	2.9	2.9	2.9	2.8	2.9	2.9	2.9	2.9	2.9	2.9	3.0
Phytase (FTU/kg)	< 60	< 60	< 60	< 60	< 60	240	580	730	330	600	1130

[1]BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

[2]Purified diatomaceous earth mainly consisting of SiO_2.

[3]Provided per kilogram of complete feed: vitamin A (retinyl acetate): 12,000 IU; vitamin D_3 (cholecalciferol): 2,500 IU; vitamin E (dl-α-tocopherol): 50 mg; vitamin K_3 (menadione): 1.5 mg; vitamin B_1 (thiamine): 2.0 mg; vitamin B_2 (riboflavine): 7.5 mg; vitamin B_6 (pyridoxine): 3.5 mg; vitamin B_{12} (cyanocobalamin): 20 μg; niacine: 35 mg; pantothenic acid: 12 mg; choline chloride: 460 mg; folic acid: 1.0 mg; biotin: 0.2 mg; iron: 80 mg, copper: 12 mg, manganese: 85 mg; zinc: 60 mg; iodine: 0.8 mg; selenium: 0.15 mg; anti-oxidant: 125 mg.

a constant P:Ca ratio. Both DCP and calcium carbonate were supplemented at the expense of diamol. The phytase products were additions to the general mixture. All experimental diets contained 5 g/kg TiO_2 as an indigestible marker and were pelleted through a 2.5-mm die. Intended concentrations of P, Ca, and phytase were confirmed by analysis (Table 1). The feed was produced by "Research Diet Services" (Hoge Maat 10, 3961 NC Wijk bij Duurstede, Netherlands).

Sampling and Measurements

The birds and feed were weighed on a pen basis before placement on d 7 and before slaughter to calculate the ADG, ADFI, and gain per feed ratio (G:F). Subsequently, the birds were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2, and then euthanized by CO_2 asphyxiation on days 21 and 22 (3 of the 6 pens from each treatment on each d). The intestinal fill was standardized by removing the feed at 2 h before slaughter, followed by 1 h *ad libitum* access to the feed. Individual BW was recorded immediately after slaughter and all the birds in a pen were sorted by weight. The right tibiotarsus (**tibia**) and foot from bird numbers 2, 5, 8, and 11 (in increasing order of BW) were excised, labeled for individual identification, and frozen at −20°C. The small intestine was excised from all the birds in each pen and the posterior half of the section between Meckel's diverticulum and 2 cm anterior to the ileo-caeco-colonic junction was identified. A small piece that was approximately 2 cm from the anterior end of this section of each bird was cut lengthwise. The digesta was gently removed with a spatula without scraping the mucosa, pooled on a pen basis, mixed, and stored on ice until it was frozen at -80°C for microbiota analysis. The remaining part of the ileum section was flushed with cold deionized water, the content pooled on a pen basis, and immediately frozen at -20°C until it was freeze-dried. The freeze-dried samples were stored in airtight containers for further analysis.

Chemical Analyses

Samples of the diets and freeze-dried digesta were ground in a vibrating cup mill (PULVERISETTE 9, Fritsch GmbH, Idar-Oberstein, Germany) to obtain a fine powder. The pulverized feed and digesta samples were analyzed for P, Ca, and TiO_2 using inductively coupled plasma optical emission spectrometry (Shastak et al., 2012b) following sulfuric and nitric acid wet digestion as described by Boguhn et al. (2009). The method of Zeller et al. (2015) was used to determine the $InsP_{3-6}$ isomers in the feed and digesta with slight modifications as described by Sommerfeld et al. (2018). The isomers were measured by high-performance ion chromatography (ICS-3000 system, Dionex, Idstein, Germany). It was not possible to separate the enantiomers using this methodology. Therefore, the results do not distinguish between the D- and L-forms. Some $InsP_3$ isomers could not be identified because standards were unavailable. The term $InsP_{3x}$ is used for the isomers Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(2,4,5)P_3 with unknown proportions because clear discrimination was not possible due to co-elution. Enzyme activities were determined by BASF SE (Ludwigshafen, Germany) using ISO EN 30024 (International Organization for Standardization, 2009).

After defrosting, the adhering tissues, cartilage caps, and fibula bones were removed from the tibiae by hand and with tap water. The feet were used including skin, claws, and all tissues below the *articulatio intertarsalis*. Subsequently, the tibia bones were pre-dried at 30°C and the feet at 60°C for 48 h in a compartment oven (VL 115, VWR International GmbH, Darmstadt, Germany). Dry matter content was determined at 103°C for 48 h (tibiae) or 72 h (feet). The ash content was determined after ignition at 600°C in a muffle furnace (L 40/11/B170, Nabertherm GmbH, Lilienthal, Germany) for 24 h (tibiae) and 48 h (feet). The bones and feet were placed in the furnace at the beginning of the 7 h heat-up period and remained in the furnace for 5 h after it.

DNA Extraction and Illumina Amplicon Sequencing

The DNA was extracted from ileal digesta samples using a FastDNA SPIN Kit for soil from MP Biomedicals (Solon, OH) according to the manufacturer's instructions. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at −20°C. Illumina library preparation was performed according to Kaewtapee et al. (2017). In brief, the V1-2 region of the 16S rRNA bacterial gene was amplified in a 20 μL reaction. Then, 1 μL of this first PCR product was used as a template in a second PCR using multiplexing and indexing primers as described previously (Camarinha-Silva et al., 2014). The amplicons were verified by agarose gel electrophoresis, purified, and normalized using a SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA). The samples were pooled and sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform. Mothur (v.1.40) was used to perform the bioinformatic pipeline (Kozich et al., 2013). Raw forward and reverse reads were assembled using the make.contigs function. Reads with ambiguous bases, more than 8 homopolymers and sequence length higher than 355 bp and lower than 250 bp were discarded using the function screen.seqs. Sequences were aligned against the Silva database version123 and further checked for chimeras using the function chimera.uchime. Reads were classified (classify.otus) using the trainset14 of RDP with a cutoff of 80. An average of 73,260 ± 2942 was obtained per sample. In order to cluster sequences into operational taxonomic units (OTU) the function cluster.split (splitmethod—classify, taxlevel—4, cutoff—0.03) was used. The function make.shared (cutoff—0.03) was then used to create a table with the information of

number of reads present on each OTU and group. The consensus taxonomy of each OTU was obtained with the function classify.otu (label=0.03). The function get.oturep (label=0.03) was used to get the fasta sequence of each OTU representative. All singletons were discarded, and samples were standardized by total. Only OTUs with an average abundance higher than 0.0001% were considered for further analysis. A total of 1093 OTUs were obtained. The closest representative was manually identified using RDP's sequence match function (version 3) (Wang et al., 2007) with the following parameters (Strain: Both; Source: Both; Size:≥1200, Quality: Good; Taxonomy: Nomenclatural; KNN matches=1). The output taxonomy table followed the defined confidence threshold cut-off value for each taxonomic level of Yarza et al. 2014: genus (94.5%), family (86.5%), order (82.0%), class (78.5%) and phylum (75.0%). A species name was given if >97% similarity was observed with the closest representative sequence. Sequences were submitted to the European Nucleotide Archive under the accession number PRJEB38381.

Calculations and Statistical Analyses

The weight of the birds and feed consumption were recorded on a pen basis. Then, the data were used to calculate ADG, ADFI, and G:F with a correction for mortality. The prececal digestibility of P and Ca, and $InsP_6$ disappearance (y_{ij}) were calculated using the following equation:

$$y_{ij}(\%) = 100 - 100 \cdot \left(\frac{Ti\ in\ feed_{ij}}{Ti\ in\ digesta_{ij}} \cdot \frac{y\ in\ digesta_{ij}}{y\ in\ feed_{ij}} \right),$$

where y_{ij} is the concentration of the respective analyte in the ij^{th} sample and Ti is the titanium concentration, both in grams per kilogram DM.

A mixed model was fitted to the performance data, P and Ca prececal digestibility, $InsP_6$ disappearance, InsP isomers, tibia, and foot ash content using the MIXED procedure in SAS for Windows (version 9.4; SAS Institute Inc., Cary, NC). The following model was chosen for all traits measured per pen:

$$y_{ij} = \mu + \alpha_i + b_j + \varepsilon_{ii},$$

where y_{ij} is the observation of the response variable, μ is the intercept, α_i represents the effect of the i-th dietary treatment, b_j is the block effect of the j-th localization within the barn, and ε_{ij} is the residual error effect of y_{ij}. Treatment effects were considered as fixed effects, whereas the localization effects within the barn (b_j) were random effects. Ash data measurements were also taken from each bird. Thus, the model was extended by adding a random pen effect (p_{ij}) as measures were taken per animal, which was in contrast to the pen data for all other traits. The model can be shown as

$$y_{ijk} = \mu + \alpha_i + b_j + p_{ij} + \varepsilon_{ijk},$$

where p_{ij} is the random effect of pen i in location j within the barn and ε_{ijk} is the error of observation y_{ijk} from the k^{th} animal within the ij^{th} pen. The heterogeneity of error variances between treatments were tested for each trait and the model with the smallest Akaike information criterion (Wolfinger, 1993) was used. A graphical check of the residuals for the normal distribution and homogeneity of variance (besides the heterogeneity accounted for by the model) was performed. Significant treatment effects were detected using a global F-test ($P \leq 0.05$). When the F-test identified treatment differences, a multiple t-test (Fisher's LSD test) was used to explore the treatments that were different.

Additionally, the treatments were factorized into supplements and their concentrations. Concentration was used as a metric variable and a regression-type model was fitted with slopes for each supplement. The latter allowed the equivalence ratios among DCP and the 2 phytases to be calculated. These ratios were calculated using linear and nonlinear regressions, where the response of each pen was plotted against the level of phytase supplementation or P originating from DCP in the experimental diets. Linearity was tested using diet-specific means as lack of fit ($\alpha = 0.05$). The MIXED and NLMIXED procedures in SAS were used to calculate the regressions. Regressions for the effects of the 3 supplements were simultaneously calculated. A common intercept was fitted for BD, NE, N, and DCP and the linear regression model used was

$$y_{ijs} = \mu + \beta_{NE} c_{NEij} + \beta_N c_{Nij} + \beta_{DCP} c_{DCPij} + b_j + e_{ij}, s$$

where μ represents the common intercept, β_{NE}, β_N, and β_{DCP} represent the 3 slopes for supplements NE, N, and DCP, respectively, and c_{NEij}, c_{Nij}, and c_{DCPij} are the concentrations of each supplement in the ij^{th} pen, respectively. When there were no significant differences between the slopes, then the model could be simplified to

$$y_{ij} = \mu + \beta c_{ij} + b_j + e_{ij},$$

where β and c_{ij} are the common slope and the concentration used in the ij^{th} pen, respectively. In both models, all other effects are defined as being analogous to previous models. For nonlinear regression, the following model was fitted:

$$y_{ij} = a_s + (\mu - a_s) \cdot \left[1 - e^{-\beta_{NE} \cdot c_{NEij}} - e^{-\beta_N \cdot c_{Nij}} - e^{-\beta_{DCP} \cdot c_{DCPij}}\right] + b_j + e_{ij},$$

where μ represents the common intercept, a_s represents the s^{th} plateau values for the 3 supplements NE, N, DCP, and all other parameters are defined as being analogous to previous models. The ratios of parameters β_{NE}, β_N, and β_{DCP} within each trait were calculated from estimates. When the regression was exponential, then the ratio directly represents the ratio due to the exponential function involved.

The block was taken as a random effect for the linear regression and as a fixed effect for the exponential regression. The models with random and fixed block effects are equivalent because the blocks were complete

(Möhring et al., 2015). The heterogeneity of error variances between supplements were tested as described for the mixed model.

Illumina amplicon sequencing data from the total microbial communities were analyzed using PRIMER (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK) (Clarke and Warwick, 2001) and a sample similarity matrix was created using the Bray-Curtis coefficient (Bray and Curtis, 1957). Similarity percentage analysis was used to calculate the average similarity within experimental treatments and to identify the genera principally responsible for the differences among treatments (Clarke and Warwick, 2001) and the PERMANOVA routine was used to study the significant differences obtained when the dietary treatments were studied using a permutation method under a reduced model followed by pairwise multiple comparison with Benjamini-Hochberg corrections in R (R core Team, 2017). Correlations were estimated using the Pearson correlation coefficient by PRISM 6 (GraphPad Software, San Diego, CA). A correlation was considered significant when $P \leq 0.05$.

RESULTS

Performance Traits

The average BW of the broiler chickens at the beginning of the experimental period (d 7) was 181 g and did not differ among treatments ($P = 0.998$, Table 2). Supplementation with DCP, NE, or N increased final BW, ADG, ADFI, and G:F in a dose-dependent manner ($P < 0.001$). The largest DCP supplementation level produced the highest values for final BW, ADG, and ADFI. The G:F was greatest at the highest supplementation levels for the 3 supplements, but there were no significant differences among the supplements. A total of 41 birds (6.8%) died during the experimental period. Most of these birds were allocated to BD (19 birds), the lowest DCP supplement level (5 birds), NE (4 birds), and N (7 birds). The remaining birds were distributed among the other treatments with a maximum of 2 dead birds per treatment.

Digestibility of P and Ca, $InsP_6$ Disappearance, and Bone Ash

Prececal P digestibility and $InsP_6$ disappearance decreased with DCP supplementation and increased with NE and N supplementation ($P < 0.001$, Table 3). The values for NE 250, N 250, and N 500 did not significantly differ from the BD values. The highest values for pcdP and $InsP_6$ disappearance were observed when the supplementation levels of the 2 phytase products were greatest, whereas the lowest value was detected at the highest supplementation level for DCP. Prececally digested P increased as the supplement levels rose. Calcium digestibility was highest in BD and with lowest N supplementation. It decreased when the feed was supplemented with DCP, NE, and N. The lowest value occurred when DCP supplementation was highest ($P < 0.001$).

The pattern for InsP isomers in the ileum digesta was similar for the 2 phytase products (Table S1). Only Ins (1,2,3,4)P_4 and Ins(1,2,3,4,5)P_5 were slightly higher in NE than in N, whereas it was the opposite for Ins (1,2,4,5,6)P_5. The $InsP_6$ concentration in ileal digesta was highest in DCP 2.9, and lowest in NE 750 and N 1000. The tibia and foot ash masses were lowest in the BD group and increased as the DCP, NE, and N levels rose ($P < 0.001$) (Table 3). The largest amount of ash was observed when the DCP supplementation level was highest. The largest NE and N supplementation levels produced the second highest ash mass values, but there was no significant difference between the phytase products. The ash concentration results were similar to the ash mass values. The BD concentrations did not significantly differ from the lowest DCP, NE, and N supplementation levels for the foot ash concentration and the lowest DCP supplementation level for the tibia ash concentration.

Slope Ratios

The regression lines for N were nonlinear for traits pcdP and $InsP_6$ disappearance, whereas the regressions for DCP and NE were linear for these traits. Therefore, linear regression lines were calculated for all supplements so that they could be compared. All the regression lines were linear for the bone ash traits (Table 4). An exponential regression was found to be the most suitable for ADG. The slope ratios (DCP/NE, DCP/N, and NE/N) differed among the traits (Figure 1). The activity of NE causing the equivalent response of 1 g P from DCP (DCP/NE) varied between 208 FTU (ADG) and 349 FTU (foot ash concentration), and the activity of N causing an equivalent response of 1 g P from DCP (DCP/N) varied from 357 FTU (ADG) to 640 FTU

Table 2. Initial BW (d 7), final BW (d 21/22), ADG, ADFI, and G:F of broiler chickens fed the experimental diets[1] (least square means and pooled SEM, $n = 6$).

Trait	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000	Pooled SEM	*P*-value ANOVA
Initial BW (g)	181	183	178	180	179	183	182	181	185	184	181	5.4	0.998
Final BW (g)	545^{e}	640^{d}	755^{c}	812^{b}	876^{a}	677^{d}	806^{b}	821^{b}	659^{d}	737^{c}	824^{b}	20.4	<0.001
ADG (g)	22^{f}	31^{e}	40^{c}	44^{b}	48^{a}	34^{d}	43^{b}	44^{b}	32^{de}	38^{c}	44^{b}	0.9	<0.001
ADFI (g)	40^{c}	48^{d}	57^{c}	62^{b}	67^{a}	51^{d}	61^{b}	62^{b}	49^{d}	56^{c}	63^{b}	1.3	<0.001
G:F (g/g)	0.57^{e}	0.65^{d}	0.70^{bc}	0.71^{ab}	0.72^{a}	0.66^{d}	0.70^{ab}	0.71^{ab}	0.65^{d}	0.68^{c}	0.70^{a}	0.006	<0.001

[1]BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.
[a–f]Means without a common superscript in each line are significantly different according to Fisher's LSD test ($\alpha = 0.05$).

Table 3. Prececal phosphorus and calcium digestibility, prececal digestible P (pcdP), $InsP_6$ disappearance ($n = 6$), and tibia and foot ash contents of broiler chickens fed the experimental diets[1] ($n = 24$; least square means and pooled SEM).

Trait	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000	Pooled SEM	*P*-value ANOVA
$InsP_6$ disappearance (%)	60^{c}	47^{d}	35^{e}	26^{f}	19^{g}	62^{c}	69^{b}	76^{a}	66^{bc}	60^{c}	76^{a}	2.3	<0.001
P digestibility (%)	58^{d}	52^{e}	46^{f}	44^{fg}	41^{g}	57^{d}	62^{bc}	66^{a}	59^{cd}	57^{d}	65^{ab}	1.3	<0.001
pcdP (g/kg DM)	2.59^{cd}	2.66^{cd}	2.67^{cd}	2.91^{ab}	3.03^{a}	2.55^{d}	2.76^{bc}	2.97^{a}	2.62^{cd}	2.53^{d}	2.92^{ab}	0.06	<0.001
pcdP intake (mg/day)	102^{g}	126^{f}	152^{d}	179^{bc}	203^{a}	129^{ef}	168^{c}	184^{b}	129^{ef}	142^{de}	183^{b}	4.6	<0.001
Ca digestibility (%)	68^{ab}	58^{d}	53^{e}	49^{f}	42^{g}	63^{c}	62^{c}	65^{bc}	70^{a}	63^{c}	65^{bc}	1.4	<0.001
Tibia ash (mg)	384^{g}	468^{f}	582^{cd}	672^{b}	783^{a}	495^{ef}	658^{b}	686^{b}	481^{ef}	528^{de}	634^{bc}	24.6	<0.001
Tibia ash (%)	34.2^{e}	35.6^{de}	39.7^{c}	42.0^{b}	44.4^{a}	36.7^{d}	40.8^{bc}	41.9^{b}	35.9^{d}	36.8^{d}	40.7^{bc}	0.74	<0.001
Foot ash (mg)	411^{f}	486^{e}	607^{c}	719^{b}	794^{a}	519^{de}	680^{b}	721^{b}	512^{e}	546^{d}	690^{b}	42.6	<0.001
Foot ash (%)	10.3^{e}	10.3^{e}	11.4^{d}	12.8^{b}	13.5^{a}	10.8^{e}	11.8^{cd}	12.5^{b}	10.5^{e}	10.6^{e}	12.4^{bc}	0.23	<0.001

[1]BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.
[a–g]Means without a common superscript in each line are significantly different according to Fisher's LSD test ($\alpha = 0.05$).

Table 4. Estimated parameters of the regressions calculated for the criteria of P evaluation[1].

Trait	Type of Regression[2]	Common intercept	Slope DCP (β_{DCP})	Slope NE(β_{NE})	Slope N(β_N)	R^2	RMSE
pcdP (mg/kg DM)	linear	2485 (40.9)	187 (25.5)	0.56 (0.098)	0.32 (0.070)	0.49	162
pcdP (mg/animal/d)	linear	103 (2.73)	34.7 (1.62)	0.11 (0.006)	0.069 (0.004)	0.90	10.2
$InsP_6$ disappearance (%)	linear	56.9 (1.51)	-13.7 (0.94)	0.024 (0.0036)	0.015 (0.0026)	0.91	6.00
Tibia ash (mg)	linear	393 (18.1)	133 (7.49)	0.42 (0.031)	0.22 (0.022)	0.58	99.1
Tibia ash (% DM)	linear	33.9 (0.37)	3.71 (0.232)	0.011 (0.0009)	0.0058 (0.0006)	0.52	2.99
Foot ash (mg)	linear	415 (16.5)	135 (7.0)	0.43 (0.030)	0.24 (0.022)	0.60	95.8
Foot ash (% DM)	linear	9.79 (0.139)	1.29 (0.084)	0.0037 (0.0003)	0.0021 (0.0002)	0.53	1.03
ADG (g/d)	exponential	22.0 (1.35)	0.50 (0.171)	0.0024 (0.00096)	0.0014 (0.00055)	0.80	3.41

[1]BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G, pcdP: prececally digestible P. Standard errors of the estimates are shown in parentheses.
[2]Linear regression equation: $y_{ij} = \mu + \beta_{NE}c_{NEij} + \beta_N c_{Nij} + \beta_{DCP}c_{DCPij} + b_j + e_{ij}$; Exponential regression equation: $y_{ij} = a_s + (\mu - a_s) \cdot [1 - e^{-\beta_{NE} \cdot c_{NEij}} - e^{-\beta_N \cdot c_{Nij}} - e^{-\beta_{DCP} \cdot c_{DCPij}}] + b_j + e_{ij}$. Plateau DCP = 55.6 (5.98), Plateau NE = 49.5 (5.43), Plateau N = 49.0 (4.99).

(tibia ash concentration). The ratio of responses between the 2 phytase products (NE/N) varied from 1.59 (pcdP intake) to 1.91 (tibia ash mass).

Microbiota Responses

An overall significant effect of treatment on ileal microbial composition was detected by the PERMANOVA analysis ($P = 0.023$). Pairwise comparisons including Benjamini Hochberg correction did not show any significant difference among the diets (Table S2). The alpha diversity calculations did not result in a significant difference when the Shannon diversity, Simpson, and Pielou's evenness indices were used (Figure S1). The average similarity among all experimental treatments ranged from 35-69% (Table S3).

The dominant genus in this study was *Lactobacillus*, with a relative abundance of between 97% in N 1000 and 84% in NE 500. The relative abundance distribution showed that OTU42 (*Lactobacillus crispatus*) and OTU32 (*Lactobacillus salivarius*) increased as the supplementation levels rose (Figure 2). In contrast, OTU65 (*Lactobacillus gallinarum*) decreased at the highest supplementation levels compared to the lower levels.

Lactobacillus crispatus (OTU42) was the most abundant single OTU and the highest value (44.5%) was observed when N supplementation was greatest (Figure 2). The lowest OTU42 abundance was found in BD (14%). This OTU was positively correlated with all performance traits, pcdP, and all ash traits ($R = 0.245 - 0.321$, $P = 0.010 - 0.050$; Figure S2, Table S4). The OTU with the second-highest abundance was assigned to *L. gallinarum* (OTU65) and its abundance was

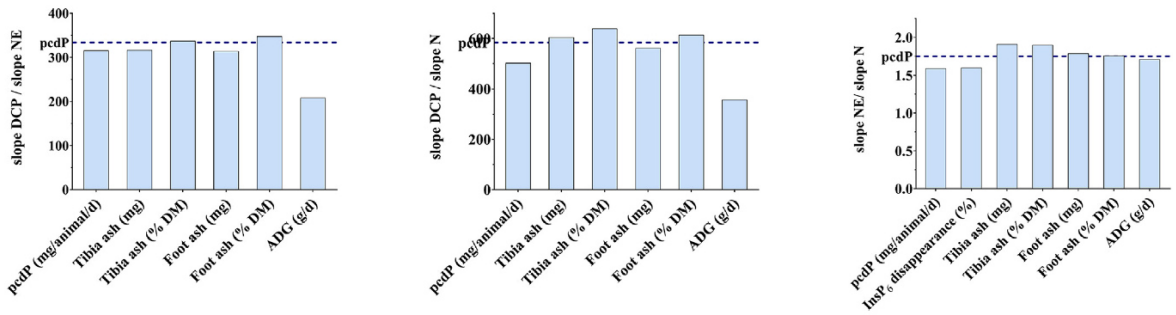

Figure 1. Equivalence calculations for the 3 supplements and the various response traits. Dotted lines show the equivalence calculated based on the concentration of prececally digestible P (pcdP) in the diet. Abbreviations: DCP, Dicalcium phosphate; NE, Natuphos E 5000 G; N, Natuphos 5000 G.

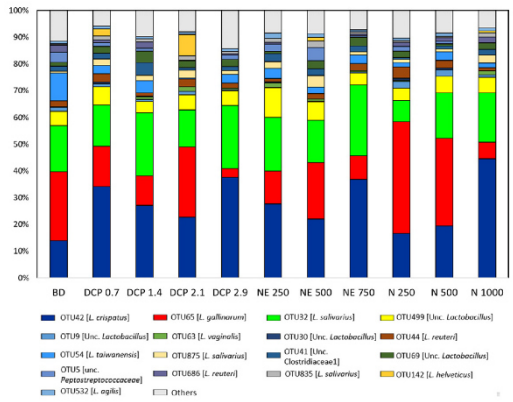

Figure 2. Relative abundance (%) of the most prevalent OTUs in the ileal content of the birds fed the experimental treatments. Abbreviations: BD, Basal diet; DCP, Dicalcium phosphate; NE, Natuphos E 5000 G; N, Natuphos 5000 G.

greatest (42%) when the N supplementation level was lowest. Operational taxonomic unit 65 had negative correlations with final BW, G:F, and the ash traits ($R = -0.236 - 0.298$, $P = 0.016 - 0.050$) (Table S4). *Lactobacillus salivarius* (OTU32) was the OTU with the third-highest abundance and was mainly present in treatment NE 750 (27%). Due to missing information in public databases, many of the sequences could not be assigned to a specific species. For example, an unclassified *Lactobacillus* (OTU499) had an abundance of 4% − 11% in treatments DCP 1.4 and NE 250, respectively. Another unclassified *Lactobacillus* (OTU9) had a very low abundance in all NE treatments (0.14 − 0.4%) and DCP 2.9 (0.4%), but its abundance increased in N 250 and N 500 (both 2.5%). Other detected species, with a maximum abundance of 4.7%, belonged to unclassified Clostridiaceae 1, *Streptococcus*, and *Enterococcus azikeevi*.

DISCUSSION

Evaluation Traits

One hypothesis of this study was that different response traits cause different estimates of relative efficacy. The results confirmed this hypothesis. Based on the suggestions made by WPSA (2013), pcdP was considered the reference trait and other traits were set in relation to pcdP. The traits providing the most consistent slope ratios with the smallest deviation from pcdP in this study were foot ash concentration and foot ash mass (Figure 1). Shastak et al. (2012a) also used foot ash mass when they undertook a regression analysis to compare different mineral P sources, but they used dissected bones without adhering tissues. Shastak et al. (2012a) found that foot ash mass was the trait with the second lowest slope ratio deviations compared to P retention after pcdP when the birds were 35 d old. In the present study, the foot contained more ash mass than the tibia in all treatments, but the variation in values was lower. Therefore, analysis of the foot may provide more accurate results than the tibia when used for phytase and P evaluation purposes. Using the foot inclusive of adhering tissues has an additional advantage over the tibia because it is less laborious to analyze.

In addition to bone data, ADG has been used as an easy-to-determine response trait in relative bioavailability studies. Denbow et al. (1995) reported that BW gain is an even more sensitive trait than tibia ash concentration for determining relative P availability. In the present study, the response ratios were very similar for ADG and pcdP when NE and N were compared, but not when the enzymes were compared to DCP (Figure 1). Létourneau-Montminy et al. (2010) reported that most of the P in an animal is retained in the bones and the amount of digestible P needed to achieve high growth performance is lower than that needed to achieve high tibia ash concentrations. This finding was confirmed by the results from the present study. An exponential function yielded the best fit for the ADG responses, whereas linear functions best fitted the pcdP and ash traits results (Table 4), which indicated that bone mineralization in the birds responded to incremental supply, although there was no further gain in BW.

The pcdP value is often expressed as a concentration (% or g/kg feed), but pcdP intake (mg/d) can also be calculated. The pcdP intake may reflect the amount of P actually retained by the animal, assuming that any excretion of P via urine is very low at the P supply level used in this study (Rodehutscord et al., 2012). However, the deviations of slope ratios of pcdP intake from pcdP concentration were ranked at positions 2−6 for the analyzed traits, which may be because the feed intake was being affected by more factors than P digestibility. This suggests that pcdP expressed as a concentration appears to be a more appropriate measure for P evaluation than pcdP intake. The mean of all response traits shown in Figure 1 leads to the conclusion that a 1.75-fold increase in the activity of phytase N is needed to achieve the same response as NE.

For $InsP_6$ disappearance, only the ratio between the 2 phytase products was calculated because supplemented DCP negatively affected prececal $InsP_6$ disappearance in a nonlinear fashion (Figure 3). Deviation from linearity only occurred at the highest DCP inclusion level. With the exception of this one treatment, the relationship between $InsP_6$-P disappearance (y, g/kg DM) and P from DCP (x, g/kg DM) was described by the following linear regression: $y = 1.72$ (*SE 0.05*) − 0.48 (*SE 0.04*)x; RMSE = 0.16, $R^2 = 0.86$), which indicates that $InsP_6$-P digestibility decreased by 0.48 g for each 1 g of supplemented DCP. This estimate is consistent with estimates from other studies where $InsP_6$-P digestibility was reduced by 0.4 to 0.5 g/kg DM for each gram of mineral P supplementation per kg of diet (Rodehutscord, 2016) and confirms the detrimental effect of using mineral P sources for phytate utilization by broiler chickens. A consistent linear relationship between $InsP_6$-P disappearance and P digestibility was found when the BD and the phytase-containing diets were analyzed together (Figure 4). The estimated slope of the

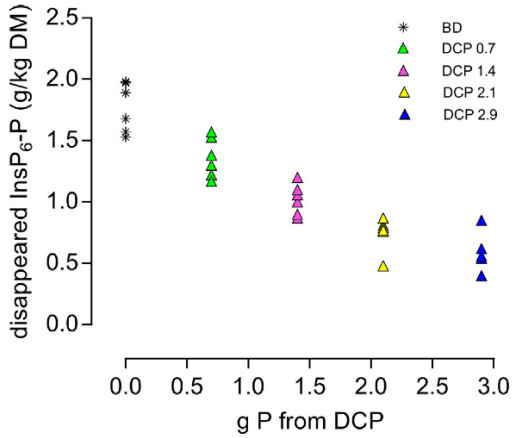

Figure 3. Relationship between the amount of supplemented P from dicalcium phosphate (DCP) and $InsP_6$-P disappearance until the end of the ileum for the basal diet (BD) and the different DCP supplementation levels. The relationship is described by the exponential function $y = 1.77\ (SE = 0.06) + (0.06\ (SE = 0.35) - 1.77\ (SE = 0.06)) \times (1 - \exp(-0.43\ (SE = 0.15)\ x)$; RMSE = 0.15, $R^2 = 0.90$.

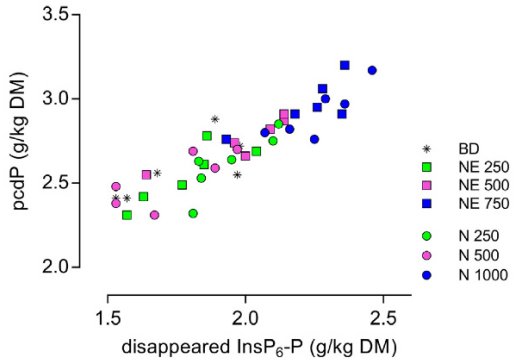

Figure 4. Relationship between $InsP_6$-P disappearance and P digestibility until the end of the ileum for the basal diet (BD), and the different supplementation levels of Natuphos E 5000 G (NE) and Natuphos 5000 G (N). Each data point represents a pen. Linear regressions were calculated together for all treatments as there were no significant differences between the single regressions ($P \geq 0.563$). The relationship is described by the linear function $y = 1.15\ (SE = 0.16) + 0.79\ (SE = 0.08)\ x$; RMSE = 0.10, $R^2 = 0.81$.

relationship indicated that 0.79 g of digestible P was provided by 1 g of $InsP_6$-P that was additionally degraded when the animal was given a phytase supplement, irrespective of the phytase product. This estimate is almost the same as the 0.78 calculated from previous studies when phytase supplements were added to several corn- and wheat-based diets (Rodehutscord, 2016). Overall, this indicates that out of all the $InsP_6$ that is initially hydrolyzed, about 20% of the P remains indigestible, probably because it is bound to lower inositol phosphates. In the present study and in previous studies from our laboratory (Zeller et al., 2015, Sommerfeld et al., 2018), ileal concentrations of some $InsP_4$ and $InsP_3$ isomers were higher in phytase-supplemented diets than in BD (Table S1). As expected, ileal concentrations of Ins(1,2,4,5,6)P_5 were higher after supplementation with N compared to NE, while the opposite was found for Ins(1,2,3,4,5)P_5, which reflected their classification as 3-phytase and 6-phytase, respectively. Differences between the 2 phytases were also observed in the ileal concentrations of Ins(1,2,3,4)P_4. However, there was no difference between the 2 phytase products with regard to the total amount of P that remained bound to lower inositol phosphates. This indicates that the differences in efficacy of the 2 phytases (Figure 1) are only related to the initial dephosphorylation of the $InsP_6$ molecule.

Ileal Microbiota Composition

The second hypothesis was that ileal microbiota composition is differentially affected by phytases and DCP. This hypothesis was not confirmed by the present data. Effects of phytase supplementation on microbiota composition due to P and Ca release from phytate have been previously reported (Witzig et al., 2015; Borda-Molina et al., 2016). In the present study, higher levels of phytases led to similar microbial ecology distributions as high concentrations of P from DCP. Bacterial species that are highly dominant in a defined environment, such as the ileum, may show reduced interactions with other microorganisms and this could improve nutrient assimilation efficiency (Larsen and Claassen, 2018).

Lactobacillus was found to be highly dominant in the present study, which was consistent with previous results (Witzig et al., 2015; Borda-Molina et al., 2016). The high dominance might be a reason for the low α-diversity. However, Witzig et al. (2015) and Borda-Molina et al. (2016) reported a higher diversity among treatments than in the present study. This may have been related to the use of other phytase products or different supplementation levels compared to the present study. Species such as *L. reuteri, L. amylovorus, L. salivarius,* and *L. crispatus,* which are known to have phytase-like activity (Raghavendra and Halami, 2009; Lee et al., 2013; Nuobariene et al., 2015; NCBI, 2016), were found in all treatments, but their proportions were not equal. However, there was no apparent connection to the supplement and supplementation levels. The abundance of *L. crispatus* was lowest in BD where a high microbial phytase-like activity was expected. Furthermore, *L. crispatus* abundance was highest in N 1000 where a decrease in microbial phytase-like activity due to high phytase supplementation was expected. Therefore, microbial phytase-like activity seemed to have a lower effect on $InsP_6$ degradation than mucosal phytase in the present study. Previous studies have suggested that among the endogenous enzymes, mucosa-derived phytases are more important than phytases produced by bacteria (Künzel et al., 2019; Sommerfeld et al., 2019).

The 2 most abundant OTUs, *L. crispatus* and *L. gallinarum*, showed significant correlations with performance and ash traits. *Lactobacillus crispatus* was positively correlated with these traits whereas *L.*

gallinarum showed negative correlations. These results were stimulated by the high inclusion levels of the respective supplements. Both strains have been reported to have functional adherence capabilities, are highly effective at colonizing the ileum, and show bile tolerance (Spivey et al., 2014). These could be compensatory effects in ecological terms and would increase the presence of the strain, possibly because of effective repeated colonization of the chicken gut due to positive responses to host genetics and environmental factors, including diet (Stephenson et al., 2010).

The conclusions of this study are that different response traits cause different estimates of relative phytase efficacy. The average values for the response traits showed that a 1.75-fold increase in the activity of phytase N is needed to achieve the same response as NE. The supplementation of DCP or either of the phytase products did not have a major effect on the microbiota composition. Similar composition was driven by the high relative abundances of *L. crispatus, L. salivarius,* and *L. gallinarum.*

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DISCLOSURES

D. F. is an employee of BASF SE. The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101133.

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Supplementary tables and figures

Relative phytase efficacy values as affected by response trait including ileal microbiota composition

by Susanne Künzel, Daniel Borda-Molina, Tobias Zuber, Jens Hartung, Wolfgang Siegert, Dieter Feuerstein, Amélia Camarinha-Silva, and Markus Rodehutscord

Table S1. Concentrations of inositol phosphate (InsP) isomers (µmol/g DM) in the digesta of broiler chickens fed the experimental diets[1] (mean and pooled standard error (SEM), n = 6).

	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE 250	NE 500	NE 750	N 250	N 500	N 1000	Pooled SEM	*P*-value ANOVA
Ins(x)P_3[2]	<LOQ	0.5^{bc}	0.9^{a}	0.7^{ab}	0.5^{bd}	0.3^{cd}	0.3^{cd}	0.4^{cd}	0.4^{cd}	0.3^{cd}	0.2^{d}	0.11	0.001
Ins(1,5,6)P_3	0.3	0.2	<LOQ	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.02	0.107
Ins(1,2,3,4)P_4	0.3^{d}	0.7^{c}	1.1^{a}	1.0^{ab}	0.8^{bc}	0.7^{c}	0.7^{c}	0.7^{c}	0.3^{d}	0.2^{d}	<LOQ	0.09	<0.001
Ins(1,2,5,6)P_4	n.d.	<LOQ	0.2^{c}	0.4^{ab}	0.4^{a}	0.2^{c}	0.2^{c}	0.2^{c}	0.2^{c}	0.3^{c}	0.3^{bc}	0.03	<0.001
Ins(1,2,3,4,6)P_5	0.5^{d}	0.7^{c}	0.8^{bc}	0.9^{b}	1.0^{a}	0.3^{ef}	0.2^{f}	<LOQ	0.4^{e}	0.4^{e}	<LOQ	0.04	<0.001
Ins(1,2,3,4,5)P_5	0.8^{d}	1.2^{c}	1.6^{b}	1.8^{b}	2.0^{a}	1.3^{c}	1.3^{c}	1.1^{c}	0.7^{d}	0.7^{d}	0.5^{e}	0.08	<0.001
Ins(1,2,4,5,6)P_5	0.5^{f}	0.9^{d}	1.2^{c}	1.7^{b}	2.2^{a}	0.6^{ef}	0.7^{ef}	0.6^{f}	0.7^{ef}	1.1^{cd}	0.9^{de}	0.08	<0.001
$InsP_6$	22.1^{d}	29.0^{c}	32.6^{bc}	36.3^{b}	40.6^{a}	19.9^{de}	15.3^{fg}	12.6^{g}	17.9^{ef}	19.7^{de}	12.4^{g}	1.34	<0.001

[1] BD: Basal diet, DCP: Dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

[2] At least one out of the following $InsP_3$ isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3.

[a-g] Means without a common superscript within one line are significantly different according to Fisher´s LSD test (α = 0.05).
Not shown isomers were not detectable (n.d.) or not quantifiable (<LOQ) in the majority of samples.

Table S2. Pairwise comparisons of the treatments[1] based on the ileal microbial composition. PERMANOVA analysis resulted in an overall *P*-value of 0.023.

	P-value	*BH correction*
BD vs DCP 0.7	0.177	0.449
BD vs DCP 1.4	0.243	0.449
BD vs DCP 2.1	0.338	0.581
BD vs DCP 2.9	0.012	0.183
BD vs NE 250	0.193	0.449
BD vs NE 500	0.471	0.755
BD vs NE 750	0.067	0.283
BD vs N250	0.239	0.449
BD vs N500	0.889	0.940
BD vs N1000	0.007	0.183
DCP 0.7 vs DCP 1.4	0.771	0.903
DCP 0.7 vs DCP 2.1	0.544	0.787
DCP 0.7 vs DCP 2.9	0.637	0.875
DCP 0.7 vs NE 250	0.740	0.903
DCP 0.7 vs NE 500	0.804	0.903
DCP 0.7 vs NE 750	0.515	0.787
DCP 0.7 vs N250	0.057	0.279
DCP 0.7 vs N500	0.241	0.449
DCP 0.7 vs N1000	0.236	0.449
DCP 1.4 vs DCP 2.1	0.224	0.449
DCP 1.4 vs DCP 2.9	0.932	0.967
DCP 1.4 vs NE 250	0.873	0.940
DCP 1.4 vs NE 500	0.747	0.903
DCP 1.4 vs NE 750	0.970	0.988
DCP 1.4 vs N250	0.044	0.253
DCP 1.4 vs N500	0.167	0.449
DCP 1.4 vs N1000	0.240	0.449
DCP 2.1 vs DCP 2.9	0.061	0.279
DCP 2.1 vs NE 250	0.222	0.449
DCP 2.1 vs NE 500	0.800	0.903
DCP 2.1 vs NE 750	0.107	0.392
DCP 2.1 vs N250	0.245	0.449
DCP 2.1 vs N500	0.725	0.903
DCP 2.1 vs N1000	0.046	0.253
DCP 2.9 vs NE 250	0.705	0.903
DCP 2.9 vs NE 500	0.480	0.755
DCP 2.9 vs NE 750	0.998	0.998
DCP 2.9 vs N250	0.013	0.183
DCP 2.9 vs N500	0.045	0.253
DCP 2.9 vs N1000	0.834	0.917

NE 250 vs NE 500	0.786	0.903
NE 250 vs NE 750	0.542	0.787
NE 250 vs N250	0.036	0.253
NE 250 vs N500	0.209	0.449
NE 250 vs N1000	0.132	0.449
NE 500 vs NE 750	0.410	0.684
NE 500 vs N250	0.189	0.449
NE 500 vs N500	0.573	0.808
NE 500 vs N1000	0.189	0.449
NE 750 vs N250	0.027	0.246
NE 750 vs N500	0.105	0.392
NE 750 vs N1000	0.262	0.466
N250 vs N500	0.670	0.898
N250 vs N1000	0.008	0.183
N500 vs N1000	0.025	0.246

[1] BD: Basal diet, DCP: Dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

Table S3. Replicates average similarity within each experimental treatment

Experimental treatment	Average similarity (%)
BD	38
DCP 0.7	52
DCP 1.4	47
DCP 2.1	47
DCP 2.9	50
NE 250	49
NE 500	35
NE750	58
N 250	50
N 500	45
N 1000	69

Table S4. Pearson correlation coefficients between the relative abundance of OTUs (minimum mean average of 0.5 %) in the ileum and other measured traits (n = 11 diets). Only significant ($P \leq 0.05$) correlations are presented.

OTU	Final BW g	ADG g	ADFI g	G:F g/g	pcdP[1] mg/kg DM	pcdP[1] mg/animal/d	Tibia ash mg	Tibia ash %	Foot ash mg	Foot ash %
OTU42	0.253	0.298	0.279	0.318	0.245	0.309	0.275	0.321	0.291	0.301
OTU65	-0.244			-0.236			-0.290	-0.298	-0.279	-0.269
OTU32										
OTU54	-0.340	-0.325	-0.353	-0.252		-0.269	-0.287		-0.307	
OTU44										
OTU686							-0.264			
OTU9	-0.271				-0.287	-0.280	-0.313	-0.364	-0.279	-0.278
OTU63	0.290						0.244		0.246	
OTU62										
OTU253					0.295					

[1] prececally digestible P

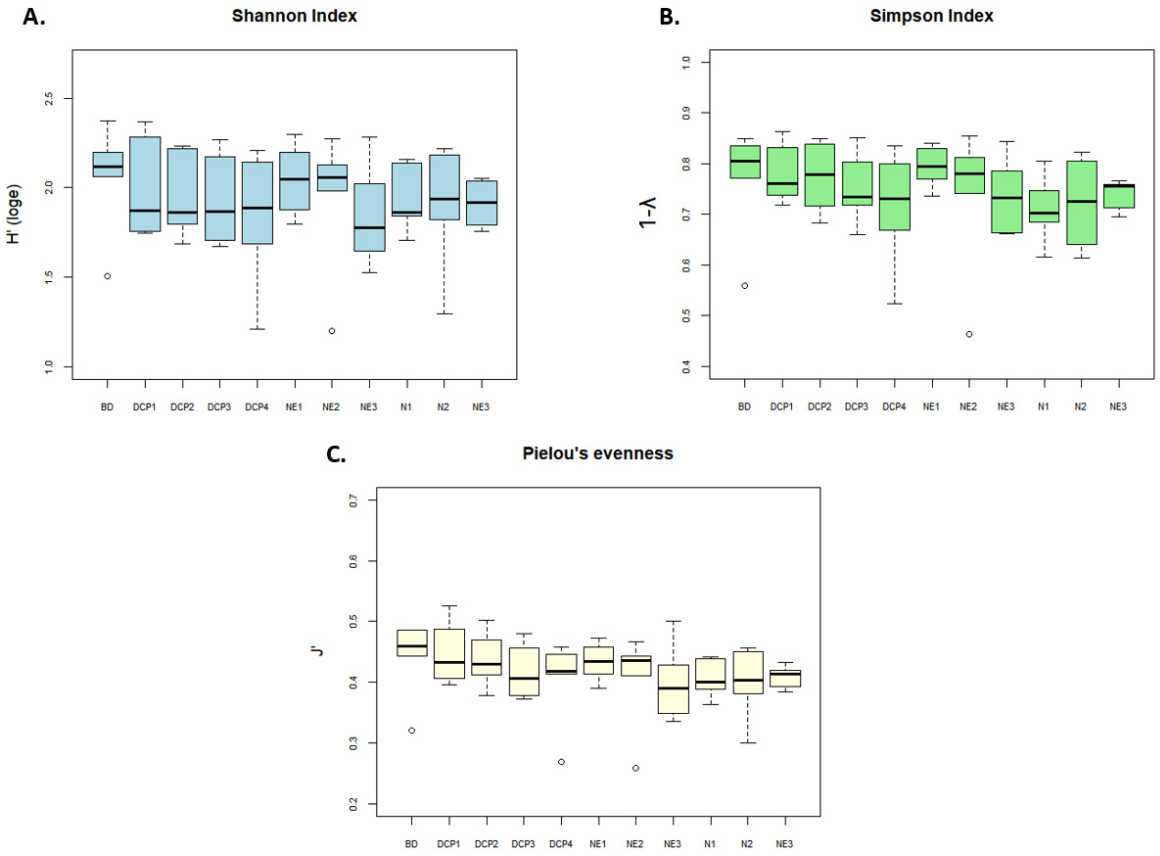

Figure S1. Box-plot for α-diversity indexes in the ileum microbiota for the experimental diets. (A) Shannon diversity, (B) Simpson Index, (C) Pielou's evenness. BD: Basal diet, DCP: Dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

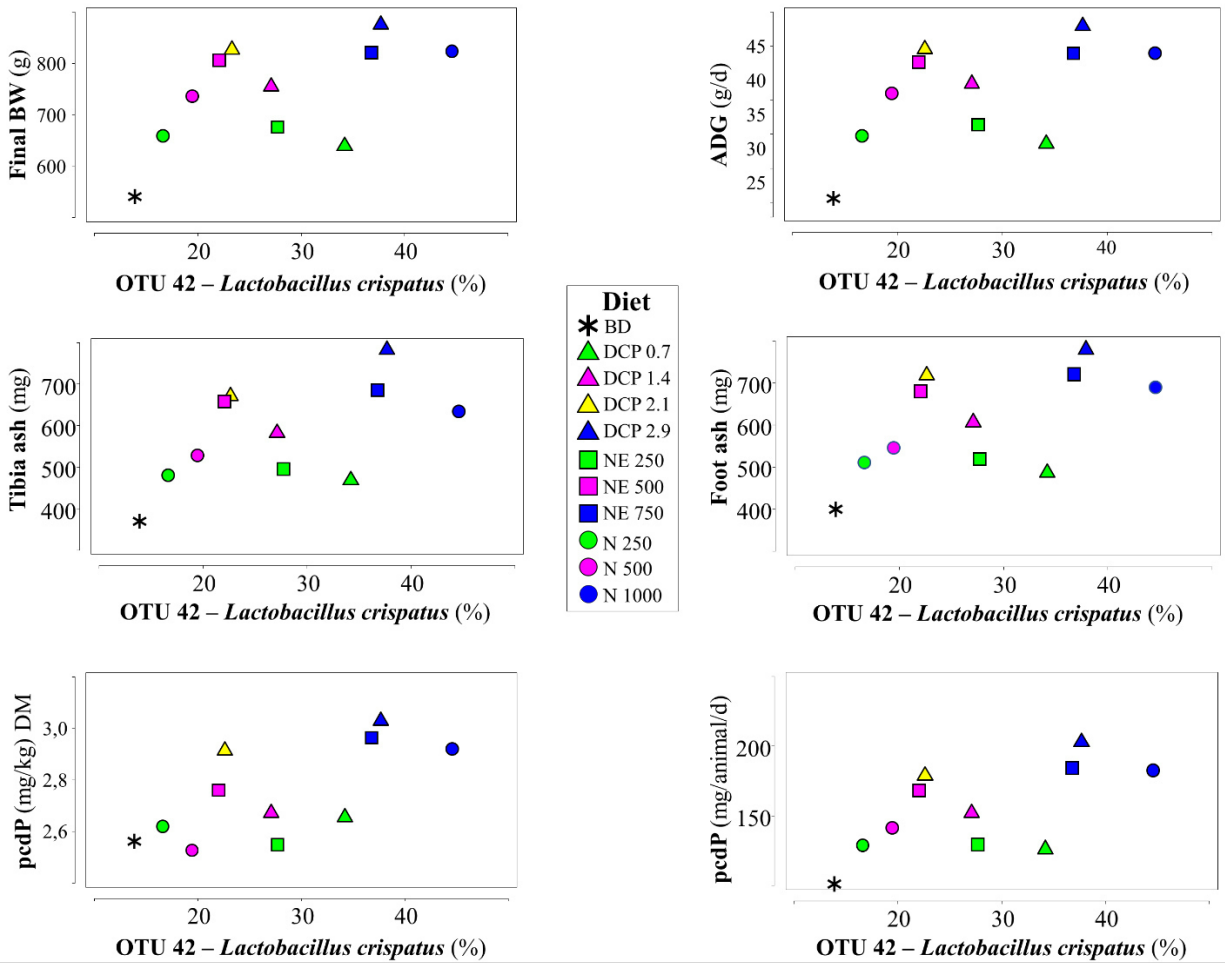

Figure S2. Relationship between relative abundance of OTU42 (assigned to *Lactobacillus crispatus*) with final BW, ADG, tibia and foot ash, prececally digestible P (pcdP) and pcdP intake in ileum digesta. BD: Basal diet, DCP: Dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

4.2 Manuscript 2

INFLUENCE OF PHYTASE OR *MYO*-INOSITOL SUPPLEMENTS ON PERFORMANCE AND PHYTATE DEGRADATION PRODUCTS IN THE CROP, ILEUM, AND BLOOD OF BROILER CHICKENS

Vera Sommerfeld[1], Susanne Künzel[1], Margit Schollenberger[1], Imke Kühn[2] and Markus Rodehutscord[1]

[1] *Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany*

[2] *AB Vista, 64293 Darmstadt, Germany*

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Influence of phytase or *myo*-inositol supplements on performance and phytate degradation products in the crop, ileum, and blood of broiler chickens

V. Sommerfeld,[*] S. Künzel,[*] M. Schollenberger,[*] I. Kühn,[†] and M. Rodehutscord[*,1]

[*]*Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany; and* [†]*AB Vista, 64293 Darmstadt, Germany*

ABSTRACT The objective of this study was to investigate the effects of supplementation with free *myo*-inositol (MI) or graded levels of phytase on inositol phosphate (InsP) degradation, concentrations of MI in the digestive tract and blood, bone mineralization, and prececal digestibility of amino acids (AA). Ross 308 broiler hatchlings were allocated to 40 pens with 11 birds each and assigned to one of 5 treatments. The birds were fed a starter diet until d 11 and a grower diet from d 11 to d 22. All diets were based on wheat, soybean meal, and corn. Birds were fed a control diet, calculated to contain adequate levels of all nutrients without (C) or with MI supplementation (C+MI), or one of 3 experimental diets that differed in phytase level (modified *E. coli*-derived 6-phytase; Phy500, Phy1500, or Phy3000 FTU/kg), with P and Ca levels adapted to the recommendations of the phytase supplier for a phytase level of 500 FTU/kg. The gain:feed ratio (G:F) was increased by MI or phytase in the starter+grower phase by 0.02 g/g. Prececal P and Ca digestibility, P and Ca concentration in blood serum, and tibia ash weight did not differ among treatments ($P > 0.05$). MI supplementation led to the highest MI concentration in the crop, ileum, and blood plasma across treatments. Phytase supplementation increased MI concentrations in the crop and ileum digesta in a dose-dependent manner and in plasma without any dose effect ($P > 0.05$). Prececal digestibility of some AA was increased by phytase. These outcomes indicate that MI might have been a relevant cause for the increase in G:F. Therefore, it is likely that the release of MI after complete dephosphorylation of phytate is one of the beneficial effects of phytase, along with the release of P and improvement in digestibility of other nutrients. Simultaneously, MI seems to have no diminishing effects on InsP degradation.

Key words: *myo*-inositol, inositol phosphate, phytate, phytase, amino acid

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INTRODUCTION

Phosphorus is predominantly bound as phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); **InsP$_6$**) and its salt, called phytate, in plant seeds (Eeckhout and de Paepe, 1994; Rodehutscord et al., 2016). In this form, it is only partially available for non-ruminant animals. Stepwise cleavage of the P groups from the phytate molecule by phytases (*myo*-inositol hexaphosphate phosphohydrolases) or phosphatases is required to make the P available. Along with their P-releasing effect, phytases also increased protein, energy, and trace mineral utilization in poultry by diminishing complexes between phytate and other nutrients (Selle and Ravindran, 2007). However, these effects were not consistent among studies.

After the theoretical complete dephosphorylation of InsP$_6$, 6 phosphate groups and *myo*-inositol (**MI**) are potentially available for absorption. Until recently, it was suggested that non-ruminants may not be able to fully degrade InsP$_6$ because 3- and 6-phytases seem to be incapable of splitting off the phosphate group in the axial position from C-atom 2 on the MI molecule (Wyss et al., 1999; Selle and Ravindran, 2007). In recent studies (Beeson et al., 2017; Sommerfeld et al., 2018), however, phytase supplementation to P-deficient and adequate diets led to higher MI concentrations in the digesta of the gizzard and ileum and the excreta of broiler chickens, and similar effects have been shown in pigs (Kühn et al., 2016). These results are a strong indication for the potential for complete InsP$_6$ degradation with subsequent MI release in the digestive tract of broiler chickens. MI is suggested to have several biological functions, e.g., playing a role in cell survival and growth, lipid metabolism, and insulin sensitivity (Huber, 2016). Recent studies assume an effect of MI on

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[1]Corresponding author: inst450@uni-hohenheim.de

growth performance when it is added in its free form to the diet or released as a result of $InsP_6$ breakdown (Zyla et al., 2013; Walk et al., 2014; Cowieson et al., 2015). However, documentation of the effects of free or phytate-released MI in poultry is scarce and often limited to a few measured traits.

Consequently, the first objective was to investigate whether supplementation with free MI results in similar responses to those of MI freed by phytase by complete InsP degradation on concentrations of MI in the digestive tract and blood plasma and performance of broiler chickens.

As P is an end product of $InsP_6$ degradation and is known for its inhibiting effect on phytases (Greiner et al., 1993; Sommerfeld et al., 2018), one might assume the same inhibitory effect of MI on inositol phosphate (**InsP**) degradation. Thus, the second objective was to investigate whether MI affects the degradation of InsPs. In addition, the possible effects of MI on prececal P, Ca, and amino acid (**AA**) digestibility and bone mineralization were investigated.

MATERIALS AND METHODS

Birds and Housing

The trial was performed at the Agricultural Experiment Station of the University of Hohenheim and approved by the Regierungspräsidium Tübingen, Germany (Project no. HOH 41/16 TE) in accordance with the German Animal Welfare Legislation. A total of 440 unsexed Ross 308 broiler hatchlings was supplied by a commercial hatchery (Brüterei Süd GmbH & Co. KG, Regenstauf, Germany) and allocated to 40 floor pens (115 × 230 cm ground area, 260 cm height) on deep litter bedding, each holding 11 hatchlings. Eight pens were randomly allocated to each of the 5 treatments in a completely randomized block design. The animals were fed a starter diet until d 11 and a grower diet from d 11 to d 22. Feed and tap water were provided for ad libitum consumption from placement to the end of the trial. From d 14 until the end, the animals were kept on perforated floors. The lighting program was as follows: 24L:0D from hatch to d 3, 22L:2D from d 4 to 7, and 15L:9D from d 8 until the end. The temperature in the barn was set at 34°C on the first d and then gradually decreased every 3 to 5 d to achieve a temperature of 26°C on the last 3 days. The well-being of the animals was checked twice daily, and the occurrence and weight of dead animals as well as the average pen feed consumption up to that day were recorded.

Diets and Treatments

All diets were based on wheat, soybean meal, and corn (Table 1). The control diet (**C**) was calculated to contain adequate levels of all nutrients according to the Nutrition Specification for ROSS 308 broilers (Aviagen, 2014). All grower diets contained 5 g/kg TiO_2 as an indigestible marker. The experimental diets included 3 phytase levels (*E.coli*-derived, modified 6-phytase; Quantum Blue®, AB Vista, UK; **Phy500**, **Phy1500**, and **Phy3000** FTU/kg feed) with P, Ca, and Na levels reduced as recommended by the phytase supplier for a level of 500 FTU/kg feed (1.5 g available P, 1.65 g Ca, and 0.3 g Na/kg feed). The fifth diet was the control diet, supplemented with 3.8 (starter) and 3.5 (grower) g/kg MI (**C+MI**). The C and C+MI diets were produced by first mixing one basal diet that was split and then supplemented with either sand or a sand and MI mixture. A similar procedure was followed for the phytase experimental diets. One basal diet was mixed, split into 3 parts, and then supplemented with individual phytase/sand mixtures to obtain the Phy500, Phy1500, and Phy3000 diets. The pelleting temperature remained below 80°C. Representative samples of each diet were ground through a 0.5 mm sieve or pulverized by a vibrating cup mill (PULVERISETTE 9, Fritsch GmbH, Idar-Oberstein, Germany), based on the analyses described in the Chemical Analysis section. Intended concentrations of Ca, P, and phytase activity were confirmed by analysis of the diets (Table 2).

Sampling and Measurements

Animals and feed were weighed on d 1, d 11, and d 22 in order to determine ADFI and to calculate ADG and the gain:feed ratio (**G:F**) on a pen basis. In order to avoid time effects, to standardize feed intake, and to ensure the filling of all birds' crops, the animals were deprived of feed for 1 h, starting 2 h before slaughter on d 22. The feeders were moved back into the pens 1 h before slaughter. One animal per pen was stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and killed by decapitation, whereupon the trunk blood was collected in tubes. Tubes containing sodium fluoride and heparin were centrifuged for 10 min at 2,000 × *g* to separate the plasma. Tubes without chemical supplements were centrifuged for 10 min at 2,000 × *g* to separate the serum. All other broilers from the same pen were stunned with the gas mixture and euthanized by CO_2 asphyxiation. Digesta from the crop and the terminal part of the ileum (last two-thirds of the section between Meckel's diverticulum and 2 cm prior to the ileo-ceco-colonic junction) of all animals from each pen were collected and pooled on a pen basis. The crop was clamped with an arterial clamp to prevent emptying and then upended to remove digesta gently with a spatula without scraping the mucosa. Digesta from the terminal ileum were rinsed with cold double-distilled water. All samples were immediately frozen at −20°C, freeze-dried, and pulverized by a vibrating cup mill. Pulverized samples were stored in

Table 1. Ingredients and calculated composition of the experimental diets.

	Starter			Grower		
Ingredient, g/kg	C	C+MI	Phy[1]	C	C+MI	Phy[1]
Wheat	460.0	460.0	476.8	500.5	500.5	517.4
Extracted soybean meal	361.7	361.7	359.3	308.1	308.1	305.8
Corn	100.0	100.0	100.0	100.0	100.0	100.0
Soybean oil	29.2	29.2	23.8	40.5	40.5	35.2
Monocalcium phosphate	16.1	16.1	9.1	14.4	14.4	7.5
Limestone	10.8	10.8	9.6	9.5	9.5	8.3
Sand	5.0	1.2	5.0/4.8/4.5	5.0	1.5	5.0/4.8/4.5
Vitamin+mineral premix[2]	4.0	4.0	4.0	4.0	4.0	4.0
Sodium bicarbonate	2.2	2.2	1.1	2.2	2.2	1.1
Sodium chloride	1.9	1.9	1.9	2.0	2.0	2.0
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	3.3	3.3	3.2	3.0	3.0	3.0
L-Lysine	2.7	2.7	2.8	2.7	2.7	2.7
L-Threonine	1.4	1.4	1.4	1.2	1.2	1.2
L-Isoleucine	0.1	0.1	0.1	0.2	0.2	0.2
L-Valine	0.4	0.4	0.4	0.4	0.4	0.4
Carbohydrase	0.2	0.2	0.2	0.2	0.2	0.2
Coccidiostat[3]	0.7	0.7	0.7	0.7	0.7	0.7
TiO_2	-	-	-	5.0	5.0	5.0
Myo-inositol	-	3.8	-	-	3.5	-
Phytase	-	-	0.1/0.3/0.6	-	-	0.1/0.3/0.6
Calculated composition, g/kg						
AMEn, kcal/kg	3000	3000	3000	3100	3100	3100
Crude Protein	235	235	235	214	214	215
Na	1.6	1.6	1.3	1.6	1.6	1.3
Ca	9.6	9.6	8.0	8.7	8.7	7.1
Total P	7.6	7.6	6.0	6.9	6.9	5.4
$InsP_6$-P	2.6	2.6	2.6	2.4	2.4	2.4

[1]Includes treatments Phy500, Phy1500, and Phy3000.

[2]Vitamin+Mineral Premix Starter (Target Feeds Limited, Shropshire, UK), provided per kg of complete diet: 13,000 IU vitamin A, 5000 IU vitamin D3, 80 IU vitamin E, 3.2 mg vitamin B1, 8.6 mg vitamin B2, 5.4 mg vitamin B6, 0.02 mg vitamin B12, 60 mg nicotinic acid, 15 mg pantothenic acid, 2.2 mg folic acid, 0.3 mg biotin, 250 mg cholinechlorid, 20 mg iron, 120 mg manganese, 9.6 mg copper, 99 mg zinc, 1.25 mg iodine, 0.3 mg selenium, 0.5 mg molybdenum.

[2]Vitamin+Mineral Premix Grower (Target Feeds Limited, Shropshire, UK), provided per kg of complete diet: 10,000 IU vitamin A, 4500 IU vitamin D3, 65 mg vitamin E, 2.5 mg vitamin B1, 6.5 mg vitamin B2, 3.2 mg vitamin B6, 0.02 mg vitamin B12, 40 mg nicotinic acid, 15 mg pantothenic acid, 1.5 mg folic acid, 0.25 mg biotin, 250 mg cholinechlorid, 20 mg iron, 120 mg manganese, 9.6 mg copper, 99 mg zinc, 1.25 mg iodine, 0.3 mg selenium, 0.5 mg molybdenum.

[3]Narasin and Nicarbazin 1:1.

airtight containers until further analysis at a temperature below 6°C.

Chemical Analysis

Ground feed samples were analyzed according to the official methods in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007) for DM (method no. 3.1).

Pulverized feed and digesta samples were analyzed for P, Ca, and Ti using the modified sulfuric and nitric acid wet digestion method of Boguhn et al. (2009) with subsequent measurement using inductively coupled plasma optical emission spectrometry, described in detail by Zeller et al. (2015a).

The extraction and measurement of $InsP_{3\ 6}$ isomers in feed and digesta were carried out using the method of Zeller et al. (2015a) with slight modifications. Samples were extracted twice with a solution of 0.2 M EDTA and 0.1 M sodium fluoride (pH of 8; 4°C) for 30 min under agitation, and were then centrifuged after each extraction at 12,000 × *g* for 15 minutes. The respective supernatants were combined and a 1-mL sample was centrifuged at 14,000 × *g* for 15 min and filtered before being centrifuged again at 14,000 × *g* for 30 minutes. Filtrates were analyzed using high-performance ion chromatography and UV detection at 290 nm after a post-column reaction with $Fe(NO_3)_3$ in $HClO_4$ using an ICS-3000 system (Dionex, Idstein, Germany). Some $InsP_3$ isomers could not be identified because the specific standards were unavailable. A clear discrimination between isomers Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(2,4,5)P_3 was not possible because of co-elution; therefore, the term $InsP_{3x}$ will be used for these $InsP_3$ isomers with unknown proportions.

For analysis of MI, samples of feed and digesta were derivatized without sample cleanup. Proteins from plasma samples were precipitated by addition of acetonitrile, and samples were lyophilized prior to derivatization. A two-step derivatization procedure comprising oximation and silanization was carried out. Deuterated MI was used as an internal standard. MI was measured using an Agilent 5977A gas chromatograph/mass spectrometer (Waldbronn, Germany).

AA analysis was performed according to Rodehutscord et al. (2004). In brief, samples were

Table 2. Analyzed composition of the experimental grower diets.

	C	C+MI	Phy500	Phy1500	Phy3000
Ca, g/kg DM	9.5	10.1	7.9	7.8	7.7
Total P, g/kg DM	7.5	8.0	6.1	5.9	5.9
$InsP_6$-P, g/kg DM	2.1	2.2	2.2	2.1	2.2
MI, μmol/g DM	1.0	14.5	1.0	1.0	2.0
Ins(1,2,3,4,6)P_5[1], μmol/g DM	0.3	0.3	0.4	0.3	0.3
Ins(1,2,3,4,5)P_5[1], μmol/g DM	0.4	0.4	0.3	0.4	0.4
Ins(1,2,4,5,6)P_5[1], μmol/g DM	0.6	0.7	0.5	0.5	0.6
$InsP_6$, μmol/g DM	11.6	11.9	11.8	11.6	12.0
Phytase Activity, FTU/kg	<50	~85	361	1870	3110
Arg, g/kg DM	14.7	15.0	15.0	14.8	14.9
His[2], g/kg DM	6.5	6.5	6.8	6.5	6.6
Ile, g/kg DM	8.9	9.1	9.2	9.1	8.7
Leu, g/kg DM	17.3	17.4	17.6	17.4	17.2
Met, g/kg DM	6.3	6.5	6.5	6.5	6.5
Phe[2], g/kg DM	11.3	11.5	11.4	11.4	11.3
Thr, g/kg DM	9.8	9.9	10.0	9.9	9.8
Val, g/kg DM	10.1	10.3	10.4	10.3	9.8

[1]No other InsP isomers were detected.
[2]The concentrations of histidine and phenylalanine may be affected to some extent by the oxidation procedure (Mason et al., 1980).

oxidized in an ice bath using a mixture of hydrogen peroxide, phenolic formic acid solution, and phenol. Then, samples were hydrolyzed at 113°C for 24 h in a mixture containing hydrochloric acid and phenol. Norleucine was used as an external standard. AA were separated and detected in an L-8900 AA analyzing system (VWR/Hitachi Ltd, Tokyo, Japan). Methionine and cysteine were determined as methionine sulfone, and cysteic acid, respectively. The concentrations of tyrosine, histidine, and phenylalanine might be affected to some extent by the oxidation procedure (Mason et al., 1980).

Ca and inorganic P ($\mathbf{P_i}$) in blood serum were analyzed at the IDEXX BioResearch Vet Med Labor GmbH (Ludwigsburg, Germany). Ca in blood serum was measured photometrically by the Arsenazo method in a Beckman Olympus AU480. P_i in blood serum was measured photometrically as phosphomolybdate complex in a Beckman Olympus AU480.

The right tibiotarsus (tibia) was removed after slaughter and stored at −20°C. Following defrosting of tibia, adhering soft tissues, cartilage caps, and fibula bones were manually removed. Bones were subsequently rinsed in distilled water and dried at 60°C for 24 h in a convection oven (VL 115, VWR International GmbH, Darmstadt, Germany). The tibia was dried for 48 h at 103°C, weighed, ashed in a muffle furnace (Nabertherm L 40/11/B170, Nabertherm GmbH, Bremen, Germany) at 600°C for 24 h, cooled in a desiccator, and weighed again. Tibia ash was determined for individual bones. We report tibia ash weight rather than ash concentration because ash weight was found to be more sensitive to dietary mineral supply than ash concentration (Shastak et al., 2012).

Feed samples were analyzed for phytase activity by Enzyme Services and Consultancy (Ystrad Mynach, UK) using the analytical method of the supplier (pH 4.5 and 60°C) followed by transfer of the result to commonly used FTU by a validated transfer factor.

Calculations and Statistical Analysis

ADG, ADFI, and G:F were calculated for the experimental periods from d 1 to d 11 (starter), d 11 to d 22 (grower), and d 1 to d 22 (starter+grower) on a pen basis and then divided by the number of remaining animals per pen. Dead animals were taken into account by calculation of their ADG and ADFI per days of life.

The disappearance of $InsP_6$ was calculated based on the analyzed concentration of $InsP_6$ and Ti in feed and digesta. The following generally accepted equation was used:

$$\text{InsP}_6 \text{ disappearance (\%)} = 100 - 100 * \left(\frac{\text{Ti in feed}}{\text{Ti in digesta}} * \frac{\text{InsP}_6 \text{ in digesta}}{\text{InsP}_6 \text{ in feed}} \right)$$

Where: Ti and $InsP_6$ are in g/kg DM. The preceal digestibility of P, Ca, CP, and AA was calculated accordingly. A correction for endogenous losses was not applied.

Digested P and Ca (y) was calculated as follows:

$$y = \frac{(\text{P or Ca digestibility} * \text{P or Ca content in feed})}{100}$$

Where: y is in g/kg DM; P or Ca digestibility is in %; and P or Ca content in feed is in g/kg DM.

All data were analyzed in a one-factorial analysis of variance using the MIXED procedure of the software package SAS (version 9.3; SAS Institute Inc., Cary, NC). For all traits analyzed in this experiment except the blood and tibia, samples were pooled on a pen basis; thus, the pen was considered as the experimental unit. In the case of the blood and the tibia data that were obtained from individual birds, the bird was considered as the experimental unit. The following model was chosen: $Y_i = \mu + \alpha_i + \varepsilon_i$, where Y_i = response variable, μ = overall mean, α_i = effect of dietary treatment, and

Table 3. Effect of phytase and *myo*-inositol supplementation on performance traits of broiler chickens.[1]

	Starter (d 1 to d 11)			Grower (d 11 to d 22)			Starter+Grower (d 1 to d 22)		
	ADG	ADFI	G:F	ADG	ADFI	G:F	ADG	ADFI	G:F
Treatment	g/d		g/g	g/d		g/g	g/d		g/g
C	26[b]	30	0.88[c,d]	77	96	0.80[b]	53	65	0.82[b]
C+MI	27[a,b]	29	0.92[a]	77	95	0.81[a]	53	64	0.84[a]
Phy500	27[a]	30	0.91[a,b]	77	95	0.82[a,b]	53	64	0.84[a]
Phy1500	26[b]	29	0.90[b,c]	78	94	0.82[a]	53	63	0.84[a]
Phy3000	25[c]	29	0.87[d]	77	94	0.82[a]	52	63	0.84[a]
Pooled SEM	0.30	0.28	0.006	1.33	1.25	0.004	0.81	0.79	0.004
P-value	<0.001	0.195	<0.001	0.996	0.822	0.037	0.933	0.504	0.005

[1]Data are given as treatment means; n = 8 pens per treatment.
[a–d]Means within a column not showing a common superscript differ ($P < 0.05$).

Table 4. Effect of phytase and *myo*-inositol supplementation on prececal Ca and P digestibility up to the terminal ileum[1], serum Ca and P_i[2], and tibia ash weight[3] of broiler chickens on d 22.

	Ca digestibility	P digestibility	Digested Ca	Digested P	Serum Ca	Serum P_i	Tibia ash weight
Treatment	%		g/kg DM		mmol/l		mg
C	45[b]	60[c]	4.3	4.6	2.98	3.48	1,201
C+MI	42[b]	58[c]	4.3	4.6	3.06	3.73	1,187
Phy500	51[a]	68[b]	4.0	4.2	3.11	3.50	1,174
Phy1500	53[a]	75[a]	4.2	4.5	3.04	3.75	1,171
Phy3000	52[a]	77[a]	3.9	4.5	2.98	3.55	1,169
Pooled SEM	2.5	1.7	0.2	0.1	0.09	0.17	16.6
P-value	<0.001	<0.001	0.319	0.064	0.599	0.663	0.555

[1]Data are given as treatment means; n = 8 pens per treatment.
[2]Data are given as treatment means; n = 8 individuals per treatment.
[3]Data are given as treatment means; n = 88 individuals per treatment.
[a–c]Means within a column not showing a common superscript differ ($P < 0.05$).

ε_i = residual error. Replicates (= blocks) were considered as random effects if significant. Correlations were calculated using the CORR procedure of SAS. Statistical significance was declared at $P < 0.05$.

RESULTS

Performance Traits

During the starter phase (d 1 to d 11), ADG was slightly increased with dietary supplementation of MI and 500 FTU/kg phytase, but not with 1,500 FTU/kg (Table 3). In treatment Phy3000, it was decreased by 1 g/d. During the grower (d 11 to d 22) and starter+grower phase (d 1 to d 22), no diet effect on ADG was found ($P = 0.996$ and $P = 0.933$, respectively). ADFI did not differ among treatments, independent of the growing phase ($P = 0.195$, $P = 0.822$, and $P = 0.504$ for starter, grower, and starter+grower, respectively). However, G:F was increased by MI and 500 FTU/kg phytase during the starter phase ($P < 0.001$). During the grower phase, G:F was increased by MI and 1,500 or 3,000 FTU/kg phytase ($P = 0.037$). G:F was increased during the starter+grower phase by MI and all phytase treatments ($P = 0.005$), but differences among phytase treatments and MI supplemented treatment were not significant.

P and Ca in the Digesta of the Terminal Ileum and Blood and Tibia Ash Weight

No effect of MI supplementation on prececal Ca or P digestibility was found (Table 4). Phytase supplementation increased prececal Ca digestibility independent of the phytase level used ($P < 0.05$). Prececal P digestibility also was increased by all phytase levels, with 1,500 and 3,000 FTU having a greater effect than 500 FTU/kg ($P < 0.05$). However, these effects on Ca and P digestibility did not translate into differences in prececally digested Ca and P, serum Ca and P_i, or tibia ash weight among dietary treatments.

Myo-inositol in Digesta and Blood Plasma

The MI concentration in crop digesta was highest in MI-fed birds (Table 5). Dietary phytase supplementation also increased the MI concentration in the crop, with 3,000 FTU/kg causing a greater increase than 500 and 1,500 FTU/kg, which did not differ from each other. The highest MI concentration in ileum

Table 5. Effect of phytase and *myo*-inositol supplementation on *myo*-inositol concentrations in crop and ileum digesta[1] and in plasma[2] of broiler chickens on d 22.

	Crop	Ileum	Plasma
Treatment	μmol/g DM		mmol/l
C	1.18[d]	3.30[e]	0.23[c]
C+MI	12.16[a]	35.90[a]	0.52[a]
Phy500	1.33[c]	10.68[d]	0.32[b]
Phy1500	1.39[c]	18.25[c]	0.36[b]
Phy3000	2.37[b]	23.76[b]	0.33[b]
Pooled SEM	0.14	1.11	0.03
P-value	<0.001	<0.001	<0.001

[1]Data are given as treatment means; n = 8 pens per treatment.
[2]Data are given as treatment means; n = 8 individuals per treatment.
[a–c]Means within a column not showing a common superscript differ ($P < 0.05$).

digesta was achieved with MI supplementation. Phytase supplementation increased the MI concentration in ileum digesta in a dose-dependent manner, but even the Phy3000 treatment did not match the C+MI treatment. Plasma MI concentration was highest in the C+MI treatment and also was increased by all phytase doses to a similar level.

InsP$_6$ Disappearance and InsP Isomers in the Crop and Terminal Ileum

Upon feeding with the control diet, 30% of the InsP$_6$ disappeared in the crop (Table 6). InsP$_6$ disappearance was not affected by MI supplementation but increased with increasing phytase supplementation. An asymptote was achieved at Phy1500, resulting in effects similar to those of Phy3000. The concentrations of the InsP$_5$ isomers and Ins(1,2,3,4)P$_4$ were lower in the treatments with phytase addition as compared to treatments C and C+MI.

InsP$_6$ disappearance up to the terminal ileum was 31 and 28% in treatments C and C+MI, respectively, and did not differ between these treatments (Table 7). InsP$_6$ disappearance was significantly increased by supplementation with 500 FTU/kg, and even more so by the 1,500 and 3,000 FTU/kg feed treatments, which did not differ from one another. The concentrations of Ins(1,2,4,5,6)P$_5$ and Ins(1,2,3,4,6)P$_5$ did not differ between treatments C and C+MI and were decreased or not detectable in the phytase-supplemented treatments. Ins(1,2,3,4,5)P$_5$ was significantly decreased in treatments Phy1500 and Phy3000. The concentrations of Ins(1,2,5,6)P$_4$ (except for Phy3000) and InsP$_{3x}$ were significantly higher and the concentration of Ins(1,2,3,4)P$_4$ significantly lower in the phytase treatments as compared to treatments C and C+MI.

Prececal Amino Acid Digestibility

The prececal digestibility of all AA was affected by dietary treatment ($P < 0.05$; Table 8 and Table 9). Dietary supplementation of MI either had no effect (His, Lys, Met, Thr, Val, Ala, Cys, Gly, Ser) or decreased prececal digestibility by 1 to 2 percentage points (Arg, Ile, Leu, Phe, Asp, Glu, Pro, Tyr). Dietary supplementation with 500 FTU/kg phytase had no effect on AA digestibility except in the case of Ile and Lys, which were increased by 1 and 2 percentage points, respectively. In treatment Phy1500, prececal digestibility of most AA was increased by 1 to 3 percentage points, with the exception of His, Thr, Cys, Gly, and Pro. Supplementation with 3,000 FTU/kg phytase increased the prececal digestibility of most AA by 1 to 3 percentage points, with the exception of His, Thr, Val, Cys, and Gly.

The digestibility of all AA was negatively correlated with the concentration of InsP$_6$ ($P < 0.03$, $-0.707 < r < -0.347$) and Ins(1,2,4,5,6)P$_5$ in the ileum ($P < 0.05$, $-0.682 < r < -0.318$). The digestibility of most AA (except Cys and His) was negatively correlated with Ins(1,2,3,4,5)P$_5$ ($P < 0.03$, $-0.615 < r < -0.352$). The digestibility of Glu, Val, Ile, Leu, Phe, Lys, and Arg was negatively correlated with the concentration of Ins(1,2,3,4,6)P$_5$

Table 6. Effect of phytase and *myo*-inositol supplementation on InsP$_6$ disappearance and concentration of InsP isomers in the crop digesta of broiler chickens on d 22.[1]

	InsP$_{3x}$[2]	Ins(1,2,3,4)P$_4$	Ins(1,2,5,6)P$_4$	Ins(1,2,3,4,6)P$_5$	Ins(1,2,3,4,5)P$_5$	Ins(1,2,4,5,6)P$_5$	InsP$_6$	InsP$_6$ disappearance
Treatment	μmol/g DM							%
C	1.1[b]	0.6[a]	0.8[c]	0.3[a]	0.7[a]	0.4[a]	7.9[a]	30[c]
C+MI	1.4[a]	0.6[a]	1.1[a,b]	0.4[a]	0.8[a]	0.4[a]	7.9[a]	31[c]
Phy500	1.5[a]	0.3[b]	1.2[a]	0.2[b]	0.4[b]	0.2[b]	6.2[b]	47[b]
Phy1500	0.8[c]	n.d.[3]	1.0[a,b]	0.2[b]	0.2[c]	0.2[b,c]	5.1[c]	56[a]
Phy3000	0.7[c]	n.d.	0.9[b,c]	<LOQ[4]	0.2[c]	0.1[c]	5.1[c]	58[a]
Pooled SEM	0.08	0.03	0.08	0.03	0.03	0.03	0.54	4.5
P-value	<0.001	<0.001	0.006	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Data are given as treatment means; n = 8 pens.
[2]At least one of the following isomers: Ins(1,2,6)P$_3$, Ins(1,4,5)P$_3$, Ins(2,4,5)P$_3$.
[3]n.d., not detectable in the majority of samples.
[4]<LOQ, not quantifiable in the majority of samples.
[a–c]Means within a column not showing a common superscript differ ($P < 0.05$).

Table 7. Effect phytase and *myo*-inositol supplementation on concentration of InsP isomers in and $InsP_6$ disappearance up to the terminal ileum of broiler chickens on d 22.[1]

	$InsP_{3x}$[2]	Ins(1,5,6)P_3	Ins(1,2,3,4)P_4	Ins(1,2,5,6)P_4	Ins(1,2,3,4,6)P_5	Ins(1,2,3,4,5)P_5	Ins(1,2,4,5,6)P_5	$InsP_6$	$InsP_6$ disappearance
Treatment				μmol/g DM					%
C	1.1[b]	n.d.[3]	1.0[a,b]	2.2[d]	0.7[a]	3.3[b]	1.4[a]	30.0[a]	31[c]
C+MI	1.4[b]	n.d.	1.2[a]	2.8[c,d]	0.7[a]	3.9[a]	1.6[a]	30.1[a]	28[c]
Phy500	2.3[a]	<LOQ[4]	0.9[b]	5.2[a]	0.2[b]	3.7[a,b]	1.1[b]	13.4[b]	70[b]
Phy1500	2.7[a]	n.d.	0.2[c]	4.2[a,b]	n.d.	0.8[c]	0.3[c]	3.3[c]	93[a]
Phy3000	2.5[a]	n.d.	n.d.	3.8[b,c]	n.d.	0.6[c]	0.2[c]	2.8[c]	94[a]
Pooled SEM	0.28	-	0.07	0.41	0.04	0.22	0.09	1.29	2.7
P-value	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Data are given as treatment means; n = 8 pens.
[2]At least one of the following isomers: Ins(1,2,6)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3.
[3]n.d., not detectable in the majority of samples.
[4]<LOQ, not quantifiable in the majority of samples.
[a–d]Means within a column not showing a common superscript differ ($P < 0.05$).

Table 8. Effect of phytase and *myo*-inositol supplementation on prececal essential AA digestibility (%) in broiler chickens on d 22.[1]

Treatment	Arg	His[2]	Ile	Leu	Lys	Met	Phe[2]	Thr	Val
C	88[b]	84[a,b]	85[c]	86[c]	87[b]	92[b]	86[b]	82[a,b]	84[b,c]
C+MI	87[c]	83[b]	84[d]	84[d]	87[b]	92[b]	85[c]	81[b]	83[c]
Phy500	89[b]	85[a]	86[b]	86[b,c]	89[a]	93[a,b]	87[b]	82[a]	85[b]
Phy1500	90[a]	85[a]	88[a]	88[a]	90[a]	93[a]	88[a]	83[a]	86[a]
Phy3000	90[a]	85[a]	87[b]	87[a,b]	90[a]	93[a]	88[a]	83[a]	85[b]
Pooled SEM	0.31	0.44	0.32	0.35	0.30	0.20	0.35	0.43	0.35
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001

[1]Data are given as treatment means; n = 8 pens.
[2]The concentrations of histidine and phenylalanine may be affected to some extent by the oxidation procedure (Mason et al., 1980).
[a–d]Means within a column not showing a common superscript differ ($P < 0.05$).

Table 9. Effect of phytase and *myo*-inositol supplementation on prececal non-essential AA digestibility (%) in broiler chickens on d 22.[1]

Treatment	Ala	Asp	Cys	Glu	Gly	Pro	Ser	Tyr[2]
C	82[b,c]	82[b]	78[a,b]	90[c]	81[a,b]	88[b]	83[b,c]	85[c]
C+MI	81[c]	81[c]	77[b]	89[d]	80[b]	87[c]	82[c]	84[d]
Phy500	83[a,b]	83[b]	78[a]	91[b,c]	81[a]	88[a,b]	84[a,b]	86[b,c]
Phy1500	84[a]	84[a]	79[a]	92[a]	82[a]	89[a,b]	85[a]	87[a]
Phy3000	84[a]	84[a]	79[a]	91[a,b]	82[a]	89[a]	85[a]	86[a,b]
Pooled SEM	0.46	0.47	0.54	0.30	0.47	0.35	0.46	0.41
P-value	0.001	<0.001	0.004	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Data are given as treatment means; n = 8 pens.
[2]The concentrations of tyrosine may be affected to some extent by the oxidation procedure (Mason et al., 1980).
[a–d]Means within a column not showing a common superscript differ ($P < 0.05$).

($P < 0.03$, $-0.640 < r < -0.447$). The digestibility of most AA (except Cys, His, and Pro) was negatively correlated with Ins(1,2,3,4)P_4 ($P < 0.03$, $-0.685 < r < -0.385$). Positive correlations were observed between the digestibility of Met, Ala, Val, Ile, Leu, His, and Lys and the concentration of Ins(1,2,5,6)P_4 ($P < 0.03$, $0.345 < r < 0.443$) and between the digestibility of most AA (except Cys, Asp, Pro, and Gly) and $InsP_{3x}$ ($P < 0.05$, $0.317 < r < 0.516$).

DISCUSSION

Effects of Myo-inositol Supplementation

The aim of this study was to investigate the effects of MI supplementation on performance traits, InsP degradation, MI concentrations in the crop, ileum, and blood, and the prececal P, Ca, and AA digestibility of broiler chickens. Previous studies on MI supplementation of

broiler or layer diets have focused on effects on performance traits or blood metabolites, but not blood MI (Żyła et al., 2004; Pirgozliev et al., 2007; Żyła et al., 2012; Cowieson et al., 2013).

As might be expected, MI supplementation increased MI concentrations in the crop and ileum digesta and blood plasma. However, no effects of MI supplementation on any other measured trait were observed. This means that MI had no impact on the degradation of $InsP_6$. This is in contrast to P, which also is released during phytate degradation and is known for its end-product inhibition effects on phytate degradation (Greiner et al., 1993; Angel et al., 2002; Olukosi and Fru-Nji, 2014; Shastak et al., 2014; Zeller et al., 2015b). As reviewed by Lee and Bedford (2016), MI is thought to be involved in bone formation and bone mineral density in mice. This was not supported by the tibia ash data obtained in the present study. However, P and Ca levels were not limiting in this study, which suggests that the benefits of MI may be more apparent under more limiting diets. It also cannot be ruled out that other criteria of bone mineralization, such as breaking strength or mineral concentration, could have shown different results.

Supplementation with MI increased G:F in the starter+grower phase as a result of its numerically lower ADFI, while maintaining the same ADG as in treatment C. As other measured traits in treatment C+MI did not differ from those in treatment C, the assumption is that MI had a direct effect on feed efficiency. Possible causes of enhanced feed efficiency as a result of MI supplementation have been described in the literature (Żyła et al., 2004; Cowieson et al., 2013; Walk et al., 2014). However, no effects of MI supplementation on broiler performance were found in a study using a different broiler strain than that used in the present study (Linares et al., 2017). MI is involved in several signaling pathways that play roles in cell survival and growth, lipid metabolism, and insulin sensitivity (Huber, 2016; Lee and Bedford, 2016). Although the numerically low increase in G:F owing to the supplementation of MI was highly significant, further studies should be conducted to confirm this outcome and to investigate the metabolic mode of action of MI in the animal.

Effects of Supplementation of Graded Levels of Phytase

Phytase supplementation led to an increased G:F, but there was no difference in phytase levels among treatments. Because the amount of prececally digested P and total tibia ash did not differ among treatments, the improvement in G:F could not have been the result of P release by phytase. Prececal AA digestibility was not increased for most AA when 500 FTU/kg were added to the diets, but increased for several AA by 1 to 3 percentage points when 1,500 or 3,000 FTU/kg were added. However, the concentration of AA in the diets was calculated to meet the birds' requirements, and the digestibility of all AA was already very high. Clearly, no G:F benefit of the additional AA digestibility was achieved with the higher phytase doses, suggesting that the birds' requirements were met, and the additional AA absorbed were likely surplus to their needs. Indeed, at over 1,200 g and a G:F of 0.84 g/g, the birds were performing almost 20% ahead of breeder target weights and 10% ahead of G:F targets. Such growth rates leave little room for improvement. It is therefore unlikely that the increase of 1 to 3 percentage points in AA digestibility resulting from the high phytase levels caused the higher G:F.

It is likely that the MI released by phytase addition, as shown by higher MI concentrations in the crop, ileum, and blood plasma, led to better feed efficiency, as suggested by the direct MI supplementation data. Phytase supplementation was previously shown to result in increased MI concentrations in the gizzard by Beeson et al. (2017) and Walk et al. (2014), in the ileum and excreta by Beeson et al. (2017), and in blood plasma of broilers by Cowieson et al. (2013).

Based on the outcomes of this study, it seems that MI released from phytate and free MI added to the diets lead to comparable effects. The accompanying metabolomics study of Huber et al. (2017) revealed increased serotonin and dopamine concentrations in blood plasma after the addition of MI to diets. Further, the authors found a positive relationship between the MI concentration in plasma and the serotonin and dopamine levels in plasma across all treatments, including those with phytase supplementation (A. Kenéz, University of Hohenheim, Germany, personal communication). This leads to the conclusion that MI, regardless of origin, is equally available for metabolic processes. The relationship between MI and serotonin and dopamine opens new avenues for study regarding MI effects, e.g., bird behavior and thus welfare.

In this study, the supplementation of 500 FTU/kg phytase to a diet reduced in P and Ca according to the assumed P equivalency of the phytase led to similar levels of prececally digested P and Ca and tibia ash weights but a higher G:F than treatment C. Thus, the assumed P equivalency of 500 FTU/kg phytase was achieved. Higher phytase levels did not further increase growth rate or efficiency.

Phytase supplementation also increased MI concentration in the crop and ileum in a dose-dependent manner, which is in agreement with Beeson et al. (2017). Supplementation with phytase increased MI concentration in the blood plasma, but there was no difference among the three phytase levels. This confirms the results of Cowieson et al. (2015), who also found higher MI concentrations in the blood plasma of broilers with phytase supplementation. In their first trial, they found a significant difference only between treatments with 1,000 and 3,000 FTU/kg phytase; no difference

between treatments supplemented with 1,000 and 2000 FTU/kg or between 2,000 and 3,000 FTU/kg phytase was found. In their second trial, no significant differences were found among treatments supplemented with 1000, 2000, or 3000 FTU/kg phytase. Based on our outcomes, we conclude that there must be an effective MI transport from the intestine into the blood. Lerner and Smagula (1979), working with incubated intestinal segments of a broiler cross, suggested that MI is transported at high substrate concentrations by diffusion. However, the ability to absorb MI varies substantially among individual birds. There may be several reasons for the similarity in blood MI levels and difference in ileum MI levels. As the plasma MI concentration of treatment C+MI was much higher than that of Phy3000, it might have been absorbed most effectively in the duodenum, and perhaps the conversion of $InsP_1$ to MI took place further down the digestive tract, whereas MI would start to be absorbed as soon as it enters the small intestine. Further, a metabolism step in the epithelial cells could have had an effect on the blood level, or perhaps the MI was transported into tissues at different speeds. However, this cannot be distinguished at this time. Further studies investigating the transport of MI in broilers and MI concentrations in different tissues would help to clarify these processes.

Relationship Between Amino Acid Digestibility and InsP Isomers

In the present study, negative correlations between the digestibility of most AA and concentrations of $InsP_6$, $Ins(1,2,4,5,6)P_5$, $Ins(1,2,3,4,5)P_5$, and $Ins(1,2,3,4)P_4$ in the ileum were found. This is in accordance to Bedford and Walk (2016), who also reported negative relationships among ileal concentrations of $InsP_6$, $InsP_5$, $InsP_4$, and $InsP_3$, without differentiation among the respective isomers, and with higher significance for $InsP_4$ and $InsP_3$. Their conclusion was that $InsP_4$ and $InsP_3$ might have a direct diminishing effect on the digestibility of several nutrients. In the present study, when no phytase was added, concentrations of $InsP_6$ and $InsP_5$ isomers were high because of limitations of endogenous microbial and epithelial phytases in degrading phytate. With phytase addition, the concentrations of $InsP_6$ and $InsP_5$ isomers were decreased and the digestibility of AA partially increased, possibly owing to diminished phytate–protein complexes or reduced endogenous losses (Selle et al., 2012). $Ins(1,2,3,4)P_4$ also was decreased, and the main $InsP_4$ isomer generated by the phytase, $Ins(1,2,5,6)P_4$, was increased. All this led to a subsequent increase in $InsP_{3x}$; thus, the positive relationship between $Ins(1,2,5,6)P_4$ and $InsP_{3x}$ and the negative relationships between $InsP_6$, $Ins(1,2,4,5,6)P_5$, $Ins(1,2,3,4,5)P_5$, and $Ins(1,2,3,4)P_4$ and AA digestibility are a logical consequence of the differences in the phytate degradation pattern. Thus, it is likely that the relationship between AA digestibility and the concentration of the respective InsP isomers is not causal but rather an effect of phytase provoking a shift in the InsP isomer pattern while simultaneously releasing AA. However, the question of whether further degradation of lower InsPs would have led to even higher AA digestibility needs to be investigated.

CONCLUSION

In the present study, a higher G:F was achieved in treatments with either phytase or MI addition. The results of all other measured traits indicate that MI might have been the main reason for this increase. Therefore, it is likely that the release of MI after complete dephosphorylation of $InsP_6$ is one of the benefits of phytase addition, independent of its effects on the release of P or the improvement in digestibility of other nutrients. MI seems to have had no effect on InsP degradation or the prececal digestibility of P, Ca, and AA. Further studies should be conducted to confirm these results.

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4.3 Manuscript 3

GENETIC PARAMETERS FOR BONE ASH AND PHOSPHORUS UTILIZATION IN AN F_2 CROSS OF JAPANESE QUAIL

Susanne Künzel, Jörn Bennewitz and Markus Rodehutscord

Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany

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Genetic parameters for bone ash and phosphorus utilization in an F_2 cross of Japanese quail

Susanne Künzel, Jörn Bennewitz, and Markus Rodehutscord[1]

Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany

ABSTRACT The main objective of this study was to perform quantitative genetic analyses of tibia and foot ash traits, which might serve as proxy traits to improve phosphorus utilization (**PU**) in a breeding program. Additionally, data for ash concentration in tibia and foot were compared with data for total amount of ash. Heritabilities for bone ash traits and genetic and phenotypic correlations between bone ash traits and PU were estimated. A total of 887 F_2 birds, established from 2 Japanese quail lines divergently selected on social reinstatement behavior, were provided a P deficient diet. In a metabolic study, feed consumption was measured and total excreta collected for each bird separately. Afterwards, birds were euthanized, the bones obtained and incinerated. Bone ash data showed a heritability of 0.230 (amount of tibia ash) to 0.342 (amount of foot ash), which was higher than estimated for PU, P retention, calcium utilization (0.120–0.174), and performance traits (0.088–0.114). The strongest genetic and phenotypic correlations between PU and bone ash traits were detected for the amount of foot ash with 0.549 and 0.527, respectively. Genetic and phenotypic correlations were stronger between PU and ash amount than between PU and ash percentage, irrespective of bone. Therefore, ash amount was considered a better trait than ash percentage to reflect PU. Strong genetic and phenotypic correlations were detected between the amount of foot and tibia ash (0.887 and 0.901, respectively). Phenotypic and genetic correlations between ash amount and PU or calcium utilization were almost identical, irrespective of bone. Foot ash is as suitable as tibia ash, but easier to determine. Bone ash data, especially the amount of foot ash, seem to be suitable indirect selection criteria for P efficiency breeding.

Key words: phosphorus utilization, heritability, tibia ash, foot ash, indirect selection trait

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INTRODUCTION

Phosphorus (**P**) is a mineral of crucial importance for all living organisms. It is needed for bone mineralization, energy metabolism, and other metabolic processes in the body. Due to globally limited availability of rock P resources and environmental impact of P contained in excreta, but also for economic reasons, it is desirable to minimize mineral P supplementation of poultry feed. *Myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) ($InsP_6$), the main storage form of P in plant seeds, is only partially available for non-ruminants (Eeckhout and Paepe, 1994; Rodehutscord et al., 2016). For this reason, poultry diets are commonly supplemented with mineral P, phytase or both.

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[1]Corresponding author: markus.rodehutscord@uni-hohenheim.de

The potential of broiler chickens for gastrointestinal $InsP_6$ degradation is high when diets have a low P concentration, indicating an endogenous phytase activity originating from the epithelial tissue or gut microbiota (Rodehutscord and Rosenfelder, 2016). Endogenous phytase activity might be affected by the genome of the animal. Japanese quail are important model organisms in poultry studies (Minvielle, 2004; Rodehutscord and Dieckmann, 2005) with the advantages of requiring less space, having a shorter generation interval and growing faster compared to other poultry species. A moderate heritability of phosphorus utilization (**PU**) was estimated, with values of 0.136 for Japanese quail (Beck et al., 2016) and 0.10 for broiler chickens (Zhang et al., 2003). However, even in quail, PU is difficult to determine under practical conditions and with a large number of animals, because it needs analysis of ileal digesta or sampling of excreta. Thus, an alternative trait that could be used for indirect selection for improved P efficiency would be beneficial. Possible traits are tibia or foot ash; both are easier to determine than the target trait PU. Tibia ash and foot ash are often used as response criteria in nutritional studies of P bioavailability in poultry (Yan et al., 2005; Shastak et al., 2012a; Shastak and Rodehutscord, 2013).

However, heritability of tibia and foot ash as well as correlations with PU are rarely known to date. Hence, the main objective of this study was to perform quantitative genetic analyses of bone ash traits and to study their interrelationship with PU and various performance traits. Additionally, the use of tibia and foot ash and the use of relative or absolute ash data were compared as criteria of bone mineralization and in genetic analysis.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted in accordance with the German Animal Welfare Legislation approved by the Animal Welfare Commissioner of the University. An F_2 cross of Japanese quail (Coturnix japonica) was used. The experimental design and procedures are described in detail by Beck et al. (2016) and the present work is an extension of that experiment. In brief, a F_2-design using two Japanese quail lines divergently selected on social reinstatement behavior was established. Selection of these founder-lines took place at INRA, Nouzilly (France) and was described by Mills and Faure (1991). In their 6th wk of life, 12 males of the F_0-generation from line A (B) were mated to 12 females from line B (A). A total of 17 roosters and 34 hens were randomly selected from the F_1-birds in their 6th week of life. One rooster was paired with two hens to generate 920 F_2-animals, whereof 887 were used for analyses following a check of plausibility of data and removal of outliers (Beck et al. 2016). The quails were generated in 12 consecutive hatches with 60–100 individuals each.

In their first 5 d of life, the F_2-animals were fed a commercial starter diet. From days 6 to 15, a low-P diet based on corn, corn starch and soybean meal was provided for ad libitum consumption. The diet did not contain a mineral P supplement or phytase. The low P content (4.0 g/kg DM) was chosen to let the birds express their full genetic potential of PU, as recommended by WPSA (2013). After 7 d of raising in groups on floor pens, F_2-birds were transferred to metabolic cages, where they were kept individually but with visual contact to neighbors. The first 2 d in these cages were for adaptation, followed by a 5-d period for phenotyping, where individual feed consumption was measured and total excreta were collected. On day 15 of age, the experiment was terminated.

Trait Measurements

Quails were weighed on days 10 and d 15 and bodyweight gain (**BWG**) was calculated as the difference between the 2 weights. Feed per gain ratio was calculated as feed intake per BWG in this period. Analyses of P and Ca in the diet and sampled excreta were done according to Shastak et al. (2012b), using an inductively coupled plasma optical emission spectrometer. For calculation of PU and calcium utilization (**CaU**), the difference between total intake and excretion during the phenotyping period for each individual was used (Beck et al., 2016).

Quails were slaughtered at the end of the phenotyping period, dissected, and tissues immediately frozen. The right tibiotarsus (tibia) of each animal was cleaned from adhering tissues, fibula bones, and cartilage caps after defrosting. The right foot of each animal was taken including skin, claws, and all tissues below the articulatio intertarsalis. Subsequently, tibiae and feet were rinsed with distilled water and dried with a lint-free paper towel. Dry matter content of tibiae and feet was determined at 103°C for 24 h in a compartment oven (VL 115, VWR International GmbH, Darmstadt, Germany). Ash content was determined after 16 h of incineration at 550°C in a muffle furnace (L 40/11/B170, Nabertherm GmbH, Lilienthal, Germany). After placement of the bones in the furnace, they were heated up for a period of 7 h and cooled down for a period of 5 h. The total amount of ash present in tibia and foot (**TA mg** and **FA mg**) as well as ash concentrations in the dry matter of bones (**TA %** and **FA %**) were considered in analyses.

Statistical Analysis

Data were analyzed using the following linear mixed model with the ASReml software (Gilmour et al., 2006):

$$y = Xb + Z_h h + Z_a a + e$$

where y = vector of observations, b = vector of the fixed overall mean, h = vector with random hatch effects, a = vector with the random additive-genetic effects of the individuals, X, Z_h and Z_a = corresponding design matrixes, and e = residual term.

The covariance structure of the random animal effect was $var\ (a) = A * \sigma_a^2$, where A = pedigree-based numerator relationship matrix and σ_a^2 = additive genetic variance. The variance of the random hatch effect was $var\ (h) = I * \sigma_h^2$, where I = identity matrix and σ_h^2 = hatch variance. The variance structure of the random residual effect was $var\ (e) = I * \sigma_e^2$, where σ_e^2 = residual variance. Heritabilities of the traits were estimated with univariate analyses. For estimation of genetic and phenotypic correlations, pairwise bivariate analyses using the same models were conducted.

RESULTS AND DISCUSSION

The genetic parameter estimates are shown in Table 1. In general, the standard errors of parameters were small for the results from the univariate analysis, but larger for bivariate analysis. This implies that the structure and size of the experiment is sufficient for the heritability estimation, but the genetic correlation

Table 1. Estimated phenotypic correlations (above the diagonal), genetic correlations (below the diagonal), and trait heritability (on the diagonal in bold), n = 887.

	TA mg	TA %	FA mg	FA %	PU	PR	CaU	BWG	FI	F:G
TA mg	**0.230**[a]	0.567*	0.901	0.510	0.511	0.753	0.646	0.584	0.740	−0.068
TA %	0.593[b]	**0.234**[a]	0.536	0.688	0.348	0.359	0.561	0.149	0.275	0.135
FA mg	0.887[a]	0.600[b]	**0.342**[a]	0.590	0.527	0.752	0.662	0.557	0.727	−0.056
FA %	0.544[b]	0.593[b]	0.687[b]	**0.310**[a]	0.268	0.293	0.530	0.009	0.211	0.277
PU	0.495[b]	0.462[c]	0.549[b]	0.464[c]	**0.134**[a]	0.789	0.839	0.599	0.552	−0.446
PR	0.866[a]	0.474[c]	0.943[a]	0.507[b]	0.705[b]	**0.120**[a]	0.694	0.837	0.932	−0.312
CaU	0.688[b]	0.619[b]	0.716[b]	0.599[b]	0.926[a]	0.776[b]	**0.174**[a]	0.370	0.478	−0.099
BWG	0.742[a]	0.177[c]	0.735[a]	0.098[c]	0.636[b]	0.905[a]	0.525[b]	**0.088**[a]	0.857	−0.652
FI	0.737[a]	0.282[c]	0.896[a]	0.307[b]	0.432[c]	0.936[a]	0.494[b]	0.869[a]	**0.114**[a]	−0.240
F:G	0.056[c]	0.198[c]	0.179[c]	0.374[c]	−0.450[c]	0.043[c]	−0.195[c]	−0.217[c]	0.238[c]	**0.110**[a]

[a]Standard error ≤ 0.10.
[b]Standard error between 0.11 and 0.20.
[c]Standard error ≥ 0.21.
*All phenotypic correlations have standard errors between 0.005 and 0.042.
Abbreviations: TA, tibia ash; FA, foot ash; PU, phosphorus utilization; PR, phosphorus retention; CaU, calcium utilization; BWG, bodyweight gain; FI, feed intake; F:G, feed per gain.

estimates have to be interpreted with some caution due to the larger standard errors.

Heritability of Bone Ash Traits

A medium heritability was detected for bone ash data with values from 0.230 (TA mg) to 0.342 (FA mg, Table 1). These estimates of heritability were higher than those estimated for PU, phosphorus retention (**PR**), and CaU (0.120–0.174) or performance traits (0.088–0.114). The heritabilities for FA mg (0.342) and FA % (0.310) were higher than those for TA mg (0.230) and TA % (0.234). Heritabilities of all bone ash traits in the present study are within the range estimated from other authors for TA in broiler chickens. For instance, heritability for TA % was 0.08 in an unselected broiler control population after feeding a diet with 7.2 g total P/kg (González-Cerón et al., 2015). Verdal et al. (2013) reported higher heritabilities of 0.41 for TA mg and 0.52 for TA % in broilers divergently selected either for high or low digestive efficiency feeding a diet with 6.6 g total P/kg.

Correlations between Bone Ash and other Traits

Standard errors of all estimated genetic correlations were large and thus have to be interpreted with some caution. The strongest genetic and phenotypic correlations between PU and bone ash traits were detected for FA mg with 0.549 and 0.527, respectively (Table 1). PU was on a high level with a mean value of 71.4%, but varied widely with a range of 21.5–87.4% (Table 2), which is partially due to different additive-genetic effects of the individuals, as can be deduced from the heritability of PU (Table 1, Beck et al. 2016). For PR, the strongest genetic correlation was detected with FA mg (0.943), and the strongest phenotypic correlation was with TA mg (0.753) and FA mg (0.752).

Table 2. Abbreviations (Abbr), mean, minimum (Min), maximum (Max), standard deviation (SD), and coefficient of variation (CV) of the observed traits of the Japanese quail F_2-animals, n = 887.

Trait	Unit	Abbr	Mean	Min	Max	SD	CV (%)
Tibia ash[1]	mg	TA mg	45.8	19.2	83.5	9.24	10
Tibia ash[1]	%	TA %	45.3	35.5	55.7	2.59	3
Foot ash[1]	mg	FA mg	44.8	19.6	83.6	8.23	11
Foot ash[1]	%	FA %	17.3	12.1	21.9	1.40	5
P utilization[2]	%	PU	71.4	21.5	87.4	8.00	4
P retention[2]	g DM	PR	0.11	0.01	0.18	0.03	8
Ca utilization[2]	%	CaU	60.6	19.4	84.3	10.0	7
Bodyweight gain[2]	g	BWG	24.5	5.8	37.9	5.04	6
Feed intake[2]	g	FI	42.7	16.1	62.4	7.13	6
Feed per gain[2]	g/g	F:G	1.78	1.21	3.92	0.30	6

[1]d15.
[2]d10–15.

The phenotypic and genetic correlations between all bone ash traits and PR were stronger than between bone ash traits and PU. PR represents the total amount of dietary P retained in the animal, and the by far highest proportion of it is retained in the bones. Therefore, bone ash has been considered a good indicator for bone mineralization and relative bioavailability of P in poultry (Shastak et al., 2012a). However, because PR is not independent from feed intake and feed intake cannot easily be determined in a large population, PU can be considered as the more suitable trait for P efficiency breeding.

Genetic and phenotypic correlations were stronger between bone ash data and CaU than between bone ash data and PU. This is likely caused by the higher amount of Ca stored in the bone compared to P. Suchý et al. (2009) reported Ca in TA to be more than twice as high as P with a P: Ca ratio of 1:2.18 in 40-d old broilers.

Comparison of Bone Ash Amount and Percentage

Genetic and phenotypic correlations were stronger between PU or PR and ash amount than ash percentage for FA and TA. Therefore, ash amount seems to be

a better trait to reflect PU and PR than ash percentage. This is in agreement with Shastak et al. (2012a) and Li et al. (2015), who reported a better reflection of bone mineralization by the use of ash amount than ash percentage. The amount of ash considers the size of the bone and therefore overall mineralization, while the relative value of ash percentage is less sensitive. Ash percentage is not a direct reflection of the Ca and P deposited in bone, since it removes bone weight differences observed between treatments (Li et al., 2015). However, most studies investigating bone mineralization have used relative values.

Comparison of Tibia and Foot Ash

The tibia is the most commonly used bone for determination of bone mineralization. However, other bones like the femur or a toe also have been used. Some authors concluded that foot ash can be used as an alternative to tibia ash in broiler chickens and recommended the use of foot ash for assessing bone mineralization (Yan et al., 2005; Garcia and Dale, 2006; Shastak et al., 2012a; Malloy et al., 2017). However, genetic analyses for foot ash were not conducted before.

In the present study, mean, minimum, and maximum values were very similar for TA mg and FA mg (Table 2) and strong genetic and phenotypic correlation existed between these 2 traits (0.887 and 0.901, respectively, Table 1). Phenotypic correlations between ash amount and PU (0.511 and 0.527 for tibia and foot, respectively), PR (0.753 and 0.752 for tibia and foot, respectively), or CaU (0.646 and 0.662 for tibia and foot, respectively) were almost identical for tibia and foot. Genetic correlations were marginally lower for TA mg than for FA mg. For ash percentage, TA showed stronger phenotypic correlations with PU, PR, and CaU than FA, genotypic correlations were almost identical. Determination of foot ash is less laborious than tibia ash. While the tibia has to be carefully defleshed before incineration, the whole food can be easily cut at the articulatio intertarsalis and does not need to be processed otherwise. Results from the present study showed that foot ash can be used as an alternative to tibia ash in determination of relative P bioavailability and genetic analysis.

In conclusion, bone ash data, especially the amount of FA, are suitable indirect selection criteria for P efficiency breeding. Bone ash traits show strong correlations with PU and PR, are easier to determine in a large number of animals, and have a higher heritability. The total amount of bone ash is a better indicator for PU and PR than bone ash concentration, and total PR values are better reflected than relative PU values. Foot ash is as suitable as tibia ash and less laborious to determine. Results of the present study should be confirmed in poultry breeding populations and using larger data sets in order to obtain smaller standard errors of the correlation coefficient estimates.

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4.4 Manuscript 4

IMPACT OF COCCIDIOSTAT AND PHYTASE SUPPLEMENTATION ON GUT MICROBIOTA COMPOSITION AND PHYTATE DEGRADATION IN BROILER CHICKENS

Susanne Künzel[1], Daniel Borda-Molina[1], Rebecca Kraft[1], Vera Sommerfeld[1], Imke Kühn[2], Amélia Camarinha-Silva[1] and Markus Rodehutscord[1]

[1] *Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany*

[2] *AB Vista, 64293 Darmstadt, Germany*

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Impact of coccidiostat and phytase supplementation on gut microbiota composition and phytate degradation in broiler chickens

Susanne Künzel[1], Daniel Borda-Molina[1], Rebecca Kraft[1], Vera Sommerfeld[1], Imke Kühn[2], Amélia Camarinha-Silva[1] and Markus Rodehutscord[1*]

Abstract

Background: There is good evidence for a substantial endogenous phytase activity originating from the epithelial tissue or the microbiota resident in the digestive tract of broiler chickens. However, ionophore coccidiostats, which are frequently used as feed additives in broiler diets to prevent coccidiosis, might affect the bacterial composition and the abundance of phytase producers in the gastrointestinal tract. The aim of the present study was to investigate whether supplementation of a frequently used mixture of the coccidiostats Narasin and Nicarbazin alone or together with a phytase affects microbiota composition of the digestive tract of broiler chickens, characteristics of phytate breakdown in crop and terminal ileum, and precaecal phosphorus and crude protein digestibility.

Results: Large differences in the microbial composition and diversity were detected between the treatments with and without coccidiostat supplementation. Disappearance of *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) ($InsP_6$) in the digestive tract, precaecal P digestibility, inorganic P in blood serum, and the concentration of inositol phosphate isomers in the crop and ileum digesta were significantly affected by phytase supplementation, but not by coccidiostat supplementation. Crude protein digestibility was increased by coccidiostat supplementation when more phosphate was available. Neither microbial abundance and diversity nor any other trait measured at the end of the experiment was affected by coccidiostat when it was only supplemented from day 1 to 10 of age.

Conclusions: The coccidiostats used herein had large effects on overall microbiota composition of the digestive tract. The coccidiostats did not seem to affect endogenous or exogenous phytase activity up to the terminal ileum of broiler chickens. The effects of phytase on growth, phosphorus digestibility, and *myo*-inositol release were not altered by the presence of the coccidiostats. The effects of phytase and coccidiostats on nutrient digestibility can be of significant relevance for phosphorus and protein-reduced feeding concepts if confirmed in further experiments.

Keywords: 16S rRNA gene, Microbiota, Coccidiostat, Phytase, Phytate, Crude protein, Broiler, Narasin, Nicarbazin

* Correspondence: inst450@uni-hohenheim.de
[1]Institut für Nutztierwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany
Full list of author information is available at the end of the article

Background

Phosphorus (P) has several essential effects in birds´ metabolism, hence it is an important element in poultry nutrition. The main ingredients of poultry diets are plant seeds and by-products from seed processing. In these feed ingredients, P is mainly bound as *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) ($InsP_6$) and its salts (phytate), and in this form only partially available to poultry. Recent studies have shown a high potential for degradation of $InsP_6$ in the digestive tract (56 – 89 %) when broiler chickens were provided with diets having low P and calcium (Ca) contents and phytate-degrading enzymes (phytase) not added [1–4]. This points towards a substantial endogenous phytase activity originating from the epithelial tissue or the microbiota resident in the digestive tract [5, 6].

A comprehensive P digestibility ring test [7] has shown that P digestibility and $InsP_6$ disappearance until the end of the ileum (precaecal) in broilers varied to a large extent between different institutions although the same experimental diets were used by all participants of the ring test and the trial protocol was standardized [8]. The authors speculated that coccidiostats used in the pre-experimental phase in some but not all institutions have contributed to this variation by influencing phytase-producing bacteria. Coccidiostats are a group of specific agents that are approved as feed additives (not meaning a medicinal product for therapy) in the EU [9, 10] for prevention of coccidiosis. Ionophore coccidiostats are known to have an effect on the microbial composition of the gastrointestinal tract [11, 12]. For example, Ludvigsen et al. reported a significant decrease of *Clostridium perfringens* in cecal content of narasin treated broilers [13]. Some microorganisms are known as phytase producers, many of them belonging to *Lactobacillus* species, such as *L. salivarius*, *L. brevis*, *L. plantarum* and *L. pentosus* [14–17]. *L. salivarius* is often found to be part of the gut microbiota of broiler chickens, specifically in the crop [18, 19]. Supplementation of antimicrobial products to poultry diets might change the proportion of phytase producing bacteria in the microbial community. A decrease of *L. salivarius* has been detected in caecal content of broiler chickens fed with diets supplemented with the antibacterial agent zinc bacitracin [20].

No study investigated effects of coccidiostats on P utilization in non-ruminants to date and only few studies investigated effects of antibacterial products. In broiler chickens and pigs, no significant effect of the antibacterial products tylosin or virginiamycin on precaecal P digestibility was found [21]. In other studies, the antibacterial agents virginiamycin and cyadox increased apparent P digestibility in pigs [22, 23]. The authors are not aware of any studies investigating the effects of in-feed antimicrobials on $InsP_6$ degradation in the digestive tract of broiler chickens and related microbiota composition.

Therefore, our objective was to study whether a widely used coccidiostat feed additive which is a mixture of a chemical (Nicarbazin) and an ionophore (Narasin) coccidiostat could affect $InsP_6$ disappearance in the digestive tract, precaecal P digestibility, and the microbiota composition. The hypothesis was that coccidiostat supplementation reduces phytate breakdown through reduction of abundance of phytase-producing bacteria. Since phytase is a frequently used feed supplement and interactions between dietary P, Ca and phytase on measured traits have been observed [4, 24], the coccidiostat effect (Coc) was investigated at different P and Ca (P/Ca) and phytase (Phy) levels. These results could help explaining variations in the results of P metabolism studies. As an additional aspect, the effect of discontinued compared to continuous coccidiostat supplementation on traits measured at the end of the experiment was investigated.

Results

Performance traits

The initial bodyweight (BW) of broiler chickens was 42.8 g on d 1, and did not differ between treatments (P = 0.805; one-way analysis of variance (ANOVA)). Bodyweight and average daily gain (ADG) at the end of the experiment were affected by the interaction between P/Ca × Phy × Coc (P = 0.033 and 0.045, respectively, Table 1). Average daily feed intake (ADFI) was increased by supplements of P/Ca, phytase, and coccidiostat with a significant P/Ca × Phy interaction (P < 0.001). Lowest values for each of the three traits were observed in treatment P/Ca-Phy-Coc-, and highest in P/Ca+Phy+Coc+. The P/Ca × Phy interaction was significant for the gain-to-feed ratio (G:F) (P < 0.001), with highest values for P/Ca+Phy+ and P/Ca-Phy+, and lowest for P/Ca-Phy-. Each of these traits behaved in a very similar way when only data recorded in phase 2 are considered. In phase 1, both phytase and coccidiostat supplementation increased ADG, ADFI, and G:F.

P, Ca and crude protein digestibility, $InsP_6$ disappearance, and foot ash

Precaecal P digestibility was increased by phytase supplementation, at P/Ca- to a greater extent (28.7 percentage points) than at P/Ca+ (17.8 percentage points), which resulted in a P/Ca × Phy interaction (P < 0.001; Table 2). Precaecal Ca digestibility was affected by the P/Ca × Coc interaction (P = 0.024), but P/Ca- showed clearly higher values for both levels of coccidiostat supplementation. Precaecal crude protein (CP) digestibility was significantly affected by an interaction between P/Ca × Phy × Coc (P = 0.050). The lowest CP digestibility was observed for treatment P/Ca-Phy+Coc- (76.2 %), and the highest for P/Ca+Phy+Coc+ (82.4 %). The combined

Table 1 Effect of the experimental diets on performance traits of broilers[1]

	Phase 1 (d1-10, *n*=14 pens)			Phase 2 (d10-24/25)			Phase 1+2 (d1-24/25)			d24/25
	ADG	ADFI	G:F	ADG	ADFI	G:F	ADG	ADFI	G:F	BW
	g/d	g/d	g/g	g/d	g/d	g/g	g/d	g/d	g/g	g
P/Ca-Phy-Coc-	-*	-	-	35^{e}	50	0.71	28^{f}	37	0.76	703^{d}
P/Ca-Phy-Coc+	-	-	-	40^{d}	56	0.72	32^{e}	41	0.77	785^{cd}
P/Ca-Phy+Coc-	-	-	-	56^{bc}	72	0.78	42^{bc}	51	0.82	1018^{bc}
P/Ca-Phy+Coc+	-	-	-	58^{b}	74	0.78	43^{b}	53	0.82	1049^{b}
P/Ca+Phy-Coc-	17	17	0.99	53^{c}	68	0.78	39^{d}	48	0.81	965^{c}
P/Ca+Phy-Coc+	18	18	1.01	54^{c}	70	0.77	40^{cd}	50	0.80	979^{c}
P/Ca+Phy+Coc-	18	18	1.02	58^{b}	73	0.79	43^{b}	52	0.82	1046^{b}
P/Ca+Phy+Coc+	19	19	1.03	64^{a}	80	0.80	47^{a}	57	0.83	1143^{a}
P/Ca-Phy-Coc±[2]	-	-	-	37	52	0.71	30	39	0.77	752
pooled SEM	0.3	0.3	0.005	1.3	1.7	0.006	1.0	1.2	0.005	26.1
					P-values					
P/Ca	-*	-	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phytase	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Coccidostat	0.001	0.006	<0.001	0.001	<0.001	0.662	0.001	<0.001	0.962	0.001
P/Ca×Phy	-	-	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P/Ca×Coc	-	-	-	0.932	0.748	0.237	0.910	0.692	0.184	0.962
Phy×Coc	0.849	0.749	0.441	0.536	0.725	0.803	0.588	0.738	0.825	0.600
P/Ca×Phy×Coc	-	-	-	0.030	0.083	0.069	0.045	0.099	0.108	0.033

[1]*ADG* average daily gain, *ADFI* average daily feed intake, *G:F* feed consumption, *BW* bodyweight; *n* = 7 pens unless otherwise stated
[2]Additional treatment, was not part of the three-factorial analysis
*Only P/Ca+ treatments in phase 1
[a–f]Means within a column not showing a common superscript differ ($P \leq 0.05$)

supplementation of phytase and coccidiostat had an increasing effect on precaecal CP digestibility at both P/Ca levels.

In the crop, $InsP_6$ disappearance was increased by phytase supplementation by 70.5 percentage points ($P < 0.001$). For the $InsP_6$ disappearance up to the terminal ileum, the P/Ca x Phy interaction was significant ($P < 0.001$) with greater disappearance found at the low P/Ca level. Coccidiostat supplementation had no effect. The highest value was observed for P/Ca-Phy+ (87.3 %), lowest for P/Ca+Phy- (12.1 %). For foot ash, either expressed as total amount or percentage, the three-way interaction was significant ($P = 0.005$ and 0.002, respectively). Both P/Ca and phytase supplementation increased food ash. Coccidiostat increased foot ash at P/Ca-, but decreased it at the higher P/Ca level in the absence of phytase.

Blood metabolites

In blood serum, inorganic phosphate (P_i) and Ca concentrations were influenced by the interaction between P/Ca × Phy, but not by coccidiostat (Table 3). The P_i concentration was increased by both P/Ca and phytase supplementation. In contrast, Ca was highest in P/Ca-Phy- and decreased upon P/Ca and phytase supplementation. Serum alkaline phosphatase (ALP) activity was decreased by P/Ca supplementation ($P < 0.001$). The significant interaction between Phy × Coc ($P = 0.044$) was caused by a decreased ALP level with supplementation of coccidiostat, phytase, or both. *Myo*-inositol (MI) in blood plasma was not affected by coccidiostat supplementation, but decreased by P/Ca (by 0.06 mmol/l; $P < 0.001$) and increased by phytase supplementation (by 0.15 mmol/l; $P < 0.001$).

Inositol phosphates and pH in crop and ileal digesta

The pH in crop digesta was not affected by the experimental diets (Additional file 1: Table S1). The concentration of $Ins(1,2,3,4,5)P_5$ in crop digesta was decreased by phytase supplementation, at P/Ca- to a higher extend than at P/Ca+, and increased by coccidiostat supplementation when phytase was not added ($P = 0.020$). Phytase supplementation decreased $InsP_6$ and $Ins(1,2,4,5,6)P_5$ concentrations, but increased concentrations of $InsP_{3x}$ and $Ins(1,2,5,6)P_4$ in crop digesta. $Ins(1,2,4,5,6)P_5$, $Ins(1,2,3,4,5)P_5$ and $Ins(1,2,5,6)P_4$ were the only $InsP_{4\text{-}5}$ analysed in crop digesta, whereas further isomers of these InsPs occurred in ileal digesta (Additional file 1: Table S2). In ileal

Table 2 Effect of the experimental diets on precaecal nutrient digestibility, $InsP_6$ disappearance[1], and foot ash[2]

	P digestibility	Ca digestibility	CP digestibility	Crop $InsP_6$ disappearance	Ileum $InsP_6$ disappearance	Foot ash	Foot ash
	%	%	%	%	%	mg	% of DM
P/Ca-Phy-Coc-	47.1	64.0	77.6^{de}	-1.7	47.7	568^{e}	9.4^{f}
P/Ca-Phy-Coc+	48.0	61.1	77.5^{de}	4.5	48.6	639^{d}	9.9^{e}
P/Ca-Phy+Coc-	76.1	62.8	76.2^{e}	71.4	88.0	1079^{c}	13.3^{d}
P/Ca-Phy+Coc+	76.4	60.1	81.6^{abc}	80.5	86.6	1074^{c}	13.1^{d}
P/Ca+Phy-Coc-	45.7	39.9	79.7^{bcd}	-0.7	11.6	1146^{b}	14.4^{b}
P/Ca+Phy-Coc+	46.5	44.6	82.1^{ab}	2.8	12.6	1077^{c}	13.9^{c}
P/Ca+Phy+Coc-	63.0	39.3	79.5^{cd}	63.9	77.3	1277^{a}	14.9^{a}
P/Ca+Phy+Coc+	64.8	39.5	82.4^{a}	70.9	76.2	1281^{a}	14.8^{a}
P/Ca-Phy-Coc±[3]	47.3	63.1	76.5	-0.1	50.3	606	9.5
pooled SEM	1.92	1.54	1.28	5.51	2.77	22.2	0.12
				P-values			
P/Ca	<0.001	<0.001	<0.001	0.286	<0.001	<0.001	<0.001
Phytase	<0.001	0.079	0.277	<0.001	<0.001	<0.001	<0.001
Coccidostat	0.489	0.854	<0.001	0.126	0.927	0.977	0.257
P/Ca×Phy	<0.001	0.437	0.282	0.327	<0.001	<0.001	<0.001
P/Ca×Coc	0.793	0.024	0.968	0.770	0.970	0.013	0.024
Phy×Coc	0.937	0.354	0.022	0.696	0.565	0.940	0.525
P/Ca×Phy×Coc	0.757	0.295	0.050	0.964	0.982	0.005	0.002

[1] *n*=7 pens
[2] *n*=70 birds
[3] Additional treatment, was not part of the three-factorial analysis
[a–e] Means within a column not showing a common superscript differ ($P \leq 0.05$)

digesta, the P/Ca × Phy interaction was significant for $InsP_6$ and $Ins(1,2,4,5,6)P_5$ ($P < 0.001$). Both were increased by P/Ca and decreased by phytase supplementation. The remaining isomers, except $Ins(1,5,6)P_3$, were increased by P/Ca supplementation. Phytase supplementation decreased $Ins(1,2,3,4,6)P_5$ and increased $Ins(1,2,5,6)P_4$ and $InsP_{3x}$ concentrations. The coccidiostat alone had no effect on InsPs degradation but increased concentration of $InsP_{3x}$ ($P = 0.049$) in combination with phytase. Ileal pH was increased by 0.4 by both P/Ca and coccidiostat supplementation. The MI concentration in ileal digesta was significantly affected by P/Ca x Phy interaction ($P = 0.013$). P/Ca supplementation reduced ileal MI concentration (by 1.3 – 2.0 g/kg DM), while phytase increased it (by 1.2 – 1.9 g/kg DM). There was a trend towards coccidiostat addition increasing ileal MI ($P = 0.090$).

Microbial communities in the crop and ileum

The microbial composition was significantly different between crop and ileum ($P = 0.001$) and between the eight dietary treatments in both crop and ileum ($P < 0.001$) (Additional file 1: Table S3A). Specifically, for both ileum and crop, the treatments supplemented with coccidiostat were significantly different from the non-supplemented ($P = 0.001$, Fig. 1a and b). The interaction section × treatment was not significant.

A total of 592 operational taxonomic units (OTUs) were shared between crop and ileum, 62 OTUs appeared only in the crop and 27 only in the ileum (Additional file 1: Figure S1A). The ten most abundant OTUs were detected in both sections and taken together they accounted for a relative abundance ranging from 95.7 % (P/Ca+Phy-Coc- ileum) to 99.1 % (P/Ca-Phy+Coc- crop). Within these OTUs, the phylum *Firmicutes* and the genus *Lactobacillus* were identified as the most abundant.

The P/Ca × Coc interaction was significant ($P = 0.008$) for the crop and the P/Ca × Phy × Coc interaction for the ileum ($P = 0.044$, Additional file 1: Table S3A). In both sections, three main groups were observed at a similarity percentage of 76 % in the crop and 70 – 76 % in the ileum (Fig. 1a and b, Additional file 1: Figure S2A and B). The first group comprised mainly samples from treatment P/Ca-Phy-Coc-, the second group all treatments supplemented with coccidiostat, and the third group consisted of treatments without coccidiostat supplementation, but with the high level of P/Ca or phytase or both. Coccidiostat supplementation resulted in a lower diversity (Shannon-Weaver index (H′)) in the microbial composition in crop and ileal digesta compared to treatments without coccidiostat (Fig. 2).

The number of OTUs differed between the treatments. In the crop, 121 (18.5 %) OTUs were detected as part of

Table 3 Effect of the experimental diets on P_i, Ca, ALP and *myo*-inositol in the blood[1]

	P_i	Ca	ALP	*Myo*-inositol
	mmol/l	mmol/l	U/l	mmol/l
P/Ca-Phy-Coc-	1.2	3.1	9239	0.33
P/Ca-Phy-Coc+	1.4	3.3	6525	0.31
P/Ca-Phy+Coc-	2.2	2.6	7368	0.48
P/Ca-Phy+Coc+	2.5	2.6	6281	0.40
P/Ca+Phy-Coc-	2.6	2.6	6753	0.23
P/Ca+Phy-Coc+	2.5	2.7	4292	0.22
P/Ca+Phy+Coc-	2.7	2.5	4745	0.39
P/Ca+Phy+Coc+	2.9	2.6	4407	0.43
P/Ca-Phy-Coc±[2]	1.2	2.9	8457	0.30
pooled SEM	0.09	0.12	657.7	0.023
			P-values	
P/Ca	<0.001	<0.001	<0.001	<0.001
Phytase	<0.001	<0.001	0.031	<0.001
Coccidostat	0.056	0.231	0.001	0.296
P/Ca×Phy	<0.001	0.001	0.904	0.071
P/Ca×Coc	0.173	0.954	0.586	0.054
Phy×Coc	0.199	0.412	0.044	0.851
P/Ca×Phy×Coc	0.515	0.397	0.787	0.082

[1] P_i: inorganic phosphate, Ca: calcium and ALP: alkaline phosphatase in blood serum; *myo*-inositol in blood plasma; *n*=14 birds
[2] Additional treatment, was not part of the three-factorial analysis

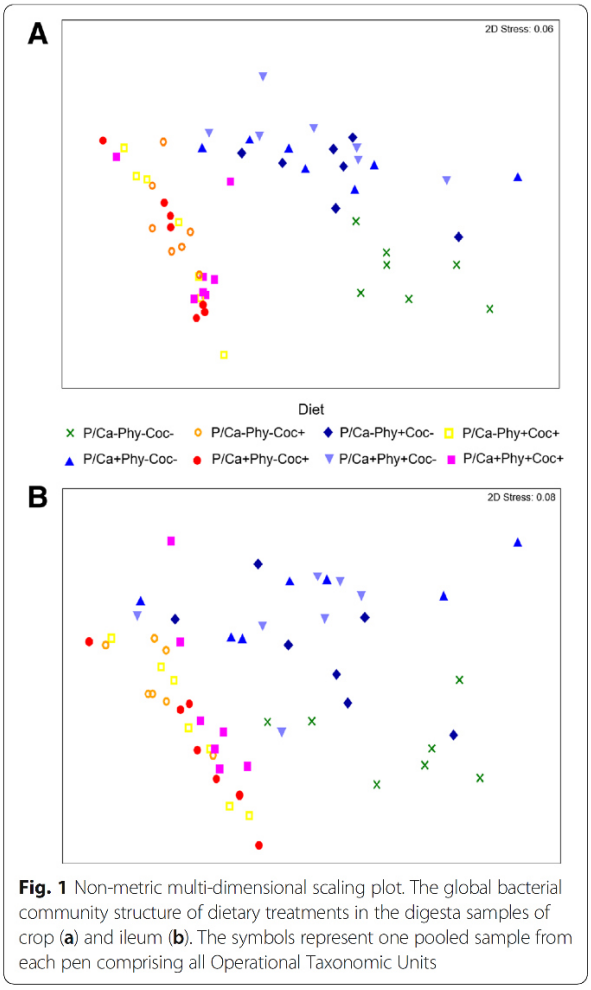

Fig. 1 Non-metric multi-dimensional scaling plot. The global bacterial community structure of dietary treatments in the digesta samples of crop (**a**) and ileum (**b**). The symbols represent one pooled sample from each pen comprising all Operational Taxonomic Units

the core microbiota shared between all treatments (Additional file 1: Figure S1B). Treatment P/Ca-Phy-Coc+ had the lowest number of OTUs (334), whereas treatment P/Ca-Phy-Coc- had the largest (416). In the ileum, 86 OTUs (13 %) were commonly present across all treatments (Additional file 1: Figure S1C). The lowest amount of detected OTUs was found in treatment P/Ca-Phy+Coc+ (264), and the highest was detected in treatment P/Ca+Phy-Coc- (333).

Lactobacillus helveticus (OTU1) was the most dominant OTU in both sections. Birds were colonized in higher abundance by this OTU when the coccidiostat was supplemented (Fig. 3). The highest abundance (56.5 % in the crop and 67.4 % in the ileum) occurred in treatment P/Ca-Phy-Coc+. Among the treatments without coccidiostat supplementation, the highest abundance of OTU1 was detected in treatment P/Ca+Phy+Coc- with 41 % in the crop and 46.7 % in the ileum. *Lactobacillus crispatus* (OTU2) was the second most abundant OTU in treatments with coccidiostat supplementation, with the highest abundance in treatment P/Ca+Phy-Coc+ (21 % in crop and 24.3 % in ileum). In treatments without coccidiostat supplementation, *Lactobacillus taiwanensis* (OTU3) was the second most abundant OTU in the crop and the ileum (with exception of P/Ca-Phy-Coc-). For this OTU, the highest abundance was observed in treatment P/Ca-Phy-Coc- with 35 % in the crop and 25 % in the ileum. The abundance of *Lactobacillus vaginalis* (OTU4) in the crop was higher in treatments with coccidiostat supplementation compared to those without. The abundance of *Lactobacillus reuteri* (OTU5) seems not to follow a specific pattern, while *Lactobacillus salivarius* (OTU7) was decreased in both ileum and crop upon coccidiostat supplementation.

Effects of discontinued coccidiostat supplementation

When birds were provided with a diet containing the coccidiostat only in phase 1 (treatment P/Ca-Phy-Coc±), they did not significantly differ from the treatment P/Ca-Phy-Coc- in any of the analyzed traits and in microbiota composition at the end of the experiment (Additional file 1: Figure S3, Table 3B and Additional file 1: Table S4, S5 and S6). However, measured P_i and Ca in blood serum, foot ash, MI in the terminal ileum, crop $InsP_6$ disappearance, and $Ins(1,2,3,4,5)P_5$ concentration were significantly lower

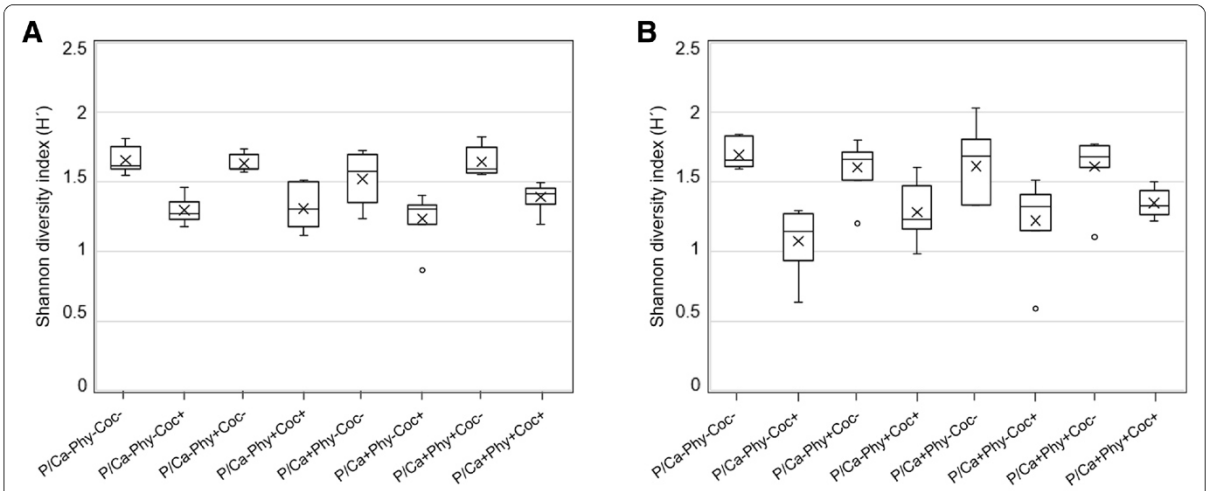

Fig. 2 Diversity measured through the Shannon diversity index. Box plots correspond to the crop (**a**) and ileum (**b**) digesta samples

in treatment P/Ca-Phy-Coc± than in P/Ca-Phy-Coc+. Microbial communities significantly differed between treatments P/Ca-Phy-Coc- or P/Ca-Phy-Coc± and P/Ca-Phy-Coc+.

The microbial communities had a similar distribution in the non-metric multidimensional scaling plots (nMDS) for the treatments P/Ca-Phy-Coc- and P/Ca-Phy-Coc±, but not for treatment P/Ca-Phy-Coc+ (Additional file 1: Figure S3). Differences in the abundance were mainly observed for OTU1 and OTU3, which were particularly dominant in crop and ileum. The abundance of *L. helveticus* (OTU1) was higher in treatment P/Ca-Phy-Coc+ (57 % in crop and 67 % in ileum) compared to P/Ca-Phy-Coc- (21 % in crop and 25 % in ileum) and P/Ca-Phy-Coc± (31 % in the crop and 35 % in ileum; Additional file 1: Figure S4). *L. taiwanensis* (OTU3) was significantly more abundant in treatments P/Ca-Phy-Coc± (31 % in crop and 20.4 % in ileum) and P/Ca-Phy-Coc- (35 % in the crop and 25 % in the ileum) than in treatment P/Ca-Phy-Coc+ (< 1 % in both crop and ileum).

Discussion

This study investigated for the first time interactive effects of coccidiostat, phytase, and P/Ca supplementation on phytate degradation and related traits. Dietary phytase supplementation increased $InsP_6$ degradation while P/Ca supplementation reduced it, which has been observed and discussed before [3, 4, 25]. Therefore, the subsequent discussion will focus on effects of the coccidiostat supplementation and the interactions with P/Ca and phytase.

Phytate degradation

The hypothesis that coccidiostat supplementation reduced the abundance of phytase producing microorganisms and thus phytate breakdown in the digestive tract of broilers has to be rejected. Substantial differences in microbiota composition between treatments were found, especially between the diets supplemented with coccidiostat or not. It is likely that the change in the composition was caused by the ionophore agent of the supplemented coccidiostat product. In contrast to nicarbazin, ionophore coccidiostats like narasin have antibacterial effects [11–13, 26]. However, coccidiostat supplementation did not significantly affect $InsP_6$ disappearance, precaecal P digestibility, P_i in blood serum, and concentration of most of the InsP isomers in crop and ileum digesta. The coccidiostat supplementation tended to increase MI concentration in the ileum, suggesting that the coccidiostat may have had an effect on intestinal phosphatases. Nevertheless, precaecal P digestibility was unaffected, indicating that any effect on phosphatases was not of much relevance.

The existence of interactions between the chicken intestinal microbiota and the diet is well described [27–29]. Nonetheless, there are only few studies using next-generation sequencing to investigate the effect of a coccidiostat on the caecal microbiota in chickens. In one study, monensin decreased the abundance of OTUs from the genus *Lactobacillus*, *Enterococcus,* and *Roseburia*, while it increased the abundance of *Coprococcus* and *Anaerofilum* [12]. Also in the present study, significant changes in some *Lactobacillus* species, which was the most abundant genus, were detected. Phytase activity has been reported to exist in different strains of *Lactobacillus, e.g., in L. plantarum, L. fermentum, L. sanfrancisensis, L. reuteri* and *L. salivarius* [14, 30–33]. In the NCBI database, a coding region comprising the phytase gene (accession Nr: KQ961566) in a strain of *L. crispatus* isolated from humans was found. In the present study, *L. helveticus* was more abundant in the crop and ileum when

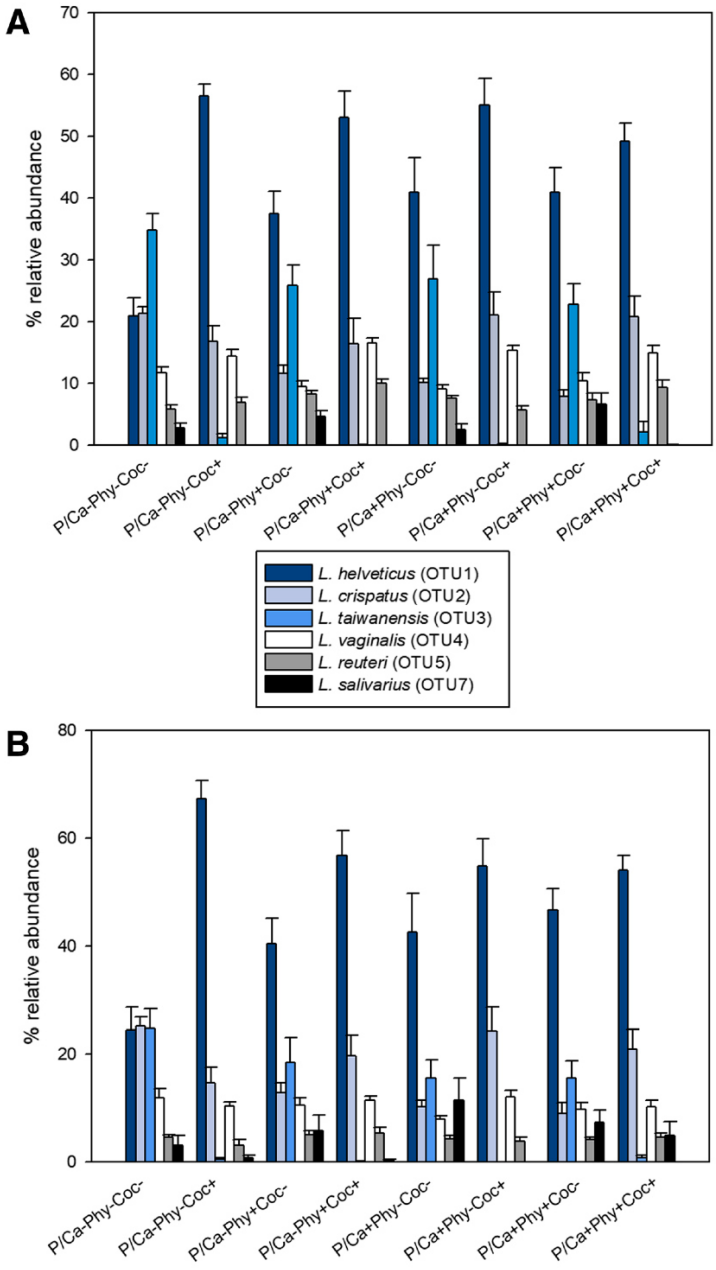

Fig. 3 Relative abundance of most abundant Operational Taxonomic Units (OTUs). The assigned OTUs correspond to *Lactobacillus* genus, found in the crop (**a**) and ileum (**b**) digesta samples. Error bars indicate standard deviation of the mean

diets contained the coccidiostat supplementation while *L. taiwanensis* and *L. salivarius* were decreased in abundance. *L. crispatus* only increased with coccidiostat supplementation at P/Ca+ and *L. vaginalis* only in the crop. The abundance of *L. reuteri* was not affected by coccidiostat supplementation. Of note, although the abundance of species with potential phytase activity was changed, this was not reflected by changes in phytate degradation. Therefore, it is possible that either the major phytase producing microorganisms were not affected by coccidiostat supplementation, or phytase producing microorganisms have generally a very restricted influence on phytate degradation, or compensatory mechanisms were involved. Potential compensatory mechanisms could be an increasing abundance of other phytase producing species to compensate for those that were decreased, an intensification of

mucosa-derived phytase activity, or a higher phytate degradation activity of those bacterial species remaining to exist in the presence of coccidiostats.

The relevance of mucosa-derived phytase for phytate degradation in the gut lumen is supposed to be low because of their localization in the brush-border membrane [5]. However, in a study with gnotobiotic broiler chickens, substantial amounts of $InsP_6$ disappeared until the end of the ileum when a low P/Ca diet without phytase supplementation was fed [6]. This points towards a high contribution of mucosa derived phytase to precaecal $InsP_6$ disappearance. In the present study, the pattern of InsP isomers was only marginally influenced by coccidiostat supplementation, suggesting that the steps of phytate degradation and involved phytase producers did not change. Perhaps, mucosa-derived phytase overall contributes more to $InsP_6$ degradation than microbiota-derived phytase as often assumed.

Another explanation for the lack of effects on $InsP_6$ disappearance could be a higher efficiency of remaining bacterial species. Species diversity was lower in the crop and ileum when the coccidiostat was supplemented. In a study using kakapo birds' faeces, microbial diversity and abundance of cellulolytic microorganisms were low, but degradation of cellulose substrates was high [34]. These authors concluded that taxonomic diversity alone does not accurately reflect the 'true' functional diversity within an ecosystem. In cows, a high feed efficiency was found to be related to a lower richness of the rumen microbiome [35]. In obese humans, low microbial diversity in faecal samples was coupled with high energy use from the food [36]. Therefore, the decrease of the microbiota diversity in the present study could have implied positive effects for the host and lead to higher phytase activity of the remaining microorganisms. Such mechanism would help to explain that the reduction in microbial diversity with coccidiostat administration was not coupled with an affect phytate degradation.

Although precaecal $InsP_6$ disappearance and P digestibility were not influenced by coccidiostat supplementation, ALP activity in blood serum was decreased by P/Ca, phytase, and coccidiostat supplementation. Increased ALP activity in the serum is associated with skeletal disorders or liver dysfunction and may be related to Ca or P deficiency or an undesirable Ca:P ratio in the diet [37, 38]. A down-regulation of this enzyme with increased availability of P has been reported [39]. This relationship was confirmed by the present study, where negative correlations between ALP activity and P_i in blood serum ($r = -0.489$, $P < 0.001$), and between ALP and foot ash amount ($r = -0.611$, $P < 0.001$) existed. It remains unclear though that the coccidiostat supplementation increased bone ash at P/Ca- but reduced it at P/Ca+. Because this coccidiostat effect disappeared in the presence of phytase, it is likely that animals might be more sensitive to P/Ca imbalances in the presence of the coccidiostats. The higher ileal pH in the presence of the coccidiostat might have reduced mineral solubility, possibly by calcium phosphate precipitation.

Crude protein digestibility

In the present study, CP digestibility values were increased by coccidiostat supplementation when more P was available, either due to phytase or mineral P supplementation. A similar effect was observed by McCormick et al. [21]. They found a significantly increased precaecal N digestibility in P deficient diets when phytase and the antibacterial products tylosin or virginiamycin were supplemented, but no single effect of phytase or the antimicrobials.

Effects of phytase supplementation on precaecal CP and amino acid (AA) digestibility exist, but effects are not consistent in literature. Explanations for phytase effect on AA digestibility include the release of protein from protein-phytate complexes and the reduction of negative impact of phytate on digestive enzymes [40]. In the present study, AAs were not analyzed, but the precaecal CP digestibility was not increased by the phytase supplementation alone. Increased CP digestibility as a result of phytase being more effective in reducing phytate-protein-complexes in the presence of coccidiostat seems unlikely. If this was the case, $InsP_6$ disappearance would increase when both products were used together. Of note, the coccidiostat supplementation significantly increased pH in ileal digesta from 6.3 to 6.7, which was highly correlated with precaecal CP digestibility ($r = 0.816$; $P < 0.001$). This increment in pH probably did not support phytate hydrolysis because the supplemented phytase acts most efficient within an optimum pH of 3.5-5.0 [41] and the pH in the lower small intestine is less relevant for phytase. Also, increased ileal pH will promote phytate precipitation which is contrasting to the observations on InsP degradation. Therefore, it is more likely that the observed coccidiostat effect was caused by a decrease in microbial protein production, leading to an increased precaecal CP digestibility. In treatments that included the coccidiostat supplementation, negative correlations between the CP digestibility and the presence of some OTUs (2, 3, 4, 5, and 7, assigned to *L. crispatus, L. taiwanensis, L. vaginalis, L. reuteri,* and *L. salivarius*) were observed. In the low P/Ca treatment without phytase, coccidiostat supplementation did not affect CP digestibility. Possibly a lack of P in the P/Ca reduced diets without phytase supplementation made it impossible for the birds to absorb more AA due to the importance of phosphate for membrane function and transporters like the Na/K-ATPase pump, which are necessary for AA absorption [42].

Another explanation for the increased CP digestibility could be a reduction of endogenous losses by coccidiostat and phytase supplementation. Coccidiostat supplementation may decrease endogenous losses for instance by extending the digesta retention time [43] or affecting the activity of digestive enzymes [44, 45]. Phytate is known to increase mucin production and endogenous AA losses [46, 47]. This effect was shown to be reduced by phytase supplementation [46, 48]. The combination of coccidiostat and phytase supplementation and the high availability of P could have led to the highest observed CP digestibility in treatment P/Ca+Phy+Coc+ resulting in the significant interaction between those supplements. Because our attempts to explain the effects on CP digestibility were speculative, more experiments should be done using different coccidiostats and phytase supplements and including AA analysis of the digesta. Further, it should be determined if the effect of coccidiostats on precaecal CP digestibility is correlated with microbial protein production.

Effect of discontinued coccidiostat supplementation

The additional treatment P/Ca-Phy-Coc± was implemented to study the effect of a coccidiostat supplement only during the starter phase on traits measured at the end of the experiment. This treatment contained the coccidiostat in phase 1, but not in phase 2 and was therefore compared to treatments P/Ca-Phy-Coc- and P/Ca-Phy-Coc+. Microbial abundance and diversity, and all other traits did not differ between the treatments P/Ca-Phy-Coc± and P/Ca-Phy-Coc- at the end of the experiment. This is remarkable considering that the early phase post-hatch is important for establishment of the gut microbial community in broiler chickens. At the time of hatching, the gastrointestinal environment is nearly sterile and with ageing, the microbial population increases and becomes more complex [44, 49]. In the present study, results indicated that the coccidiostat supplementation in phase 1 did not influence the microbial composition, or that the microbiota has adapted during phase 2 after the removal of the coccidiostat. Coccidiostat supplementation increased performance traits during phase 1. This may be seen to contradict the view of the coccidiostat not having an effect in early stage post hatch.

Significant differences in microbiota composition existed between treatments P/Ca-Phy-Coc± and P/Ca-Phy-Coc+, but not between P/Ca-Phy-Coc± and P/Ca-Phy-Coc-. For some other traits, in particular ADG, ADFI, and BW during the whole trial, treatment P/Ca-Phy-Coc± was not significantly different from the other two treatments. This indicates again towards an adaptation of the microbiota. A similar observation was made in a pig study, where precaecal AA digestibility was increased when virginiamycin was added to the diets, potentially induced by changes in the microbiota [50]. After removal of virginiamycin from the diet, this effect did no longer exist.

Based on our results, it is not expected that a mixture of Narasin and Nicarbazin in the early phase of the experiment has an influence on microbiota and P digestibility at the end of the experiment.

Conclusions

We conclude that a mixture of the coccidiostats Narasin and Nicarbazin had no discernible effect on endogenous phytase activity in the digestive tract anterior to the caeca. Coccidiostat supplementation changed the microbial distribution and diversity in the digestive tract of broilers, but did not affect phytate breakdown. Coccidiostat supplementation confined to the early phase of the experiment had no influence on microbiota and P digestibility at the end of the experiment. Crude protein digestibility was increased by coccidiostat supplementation when more P was available. Effects of supplemented phytase were not influenced by coccidiostat supplementation. These results should be verified using other coccidiostats. Further work is needed to investigate if the effect of coccidiostats on precaecal CP digestibility is correlated with microbial protein production. The influence of microbiota-derived phytase on phytate degradation processes needs to be elucidated.

Methods

Birds and housing

The trial was performed in accordance with the German Animal Welfare Legislation, approved by the Regierungspräsidium Tübingen, Germany (project no. HOH 46/17 TE) and conducted at the Agricultural Experiment Station of the University of Hohenheim. A total of 630 male Ross 308 broiler hatchlings were supplied by a commercial hatchery (*Brüterei Süd GmbH & Co. KG*, Regenstauf, Germany) and assigned to one of 9 treatments with 7 pens each in a completely randomized block design. Each floor pen (115 × 230 cm ground area, 260 cm high) was stocked with 10 hatchlings. Feed and tap water were provided for *ad libitum* consumption during the whole trial. Birds were kept on deep litter bedding until d 14. From then they were kept on perforated floors until the end to avoid an intake of litter or excreta that contain the indigestible marker. The lighting program was 24 h light : 0 h darkness until d 3, and from then 18 h light : 6 h darkness. The temperature was set at 34 °C on the day of placement and continuously decreased to achieve a temperature of 26 °C on the last day. The well-being of the animals was checked at least twice daily.

Diets and treatments

Birds were fed corn-soybean meal-based diets in 2 phases (day 1-10 and 10-25). Diets were based on the recommendations of the Gesellschaft für Ernährungs-physiologie (GFE, 1999) [51] with the exception of P and Ca in phase 2 (Table 4). The experiment was designed as a 2 × 2 × 2 + 1-factorial arrangement of treatments. It included diets without (P/Ca–, 4.2 g P and 6.5 g Ca/kg DM in phase 2) or with monocalcium phosphate and adjusted limestone supplementation (P/Ca+, 7.0 g P and 10.4 g Ca/kg DM in phase 2), without (Coc-) or with coccidiostat supplementation (Coc+, 50 mg/kg of Narasin and Nicarbazin each; Maxiban®, Elanco, Greenfield, USA), and without (Phy–) or with a modified, *E. coli*-derived 6-phytase (Phy+, 1,500 FTU/kg feed; Quantum Blue™, AB Vista, Marlborough, UK). In phase 1, P/Ca concentrations of all diets were according to the recommendations of GfE (1999) [51]. Coccidiostat and phytase were supplemented continuously to the respective diets in phase 1 and 2. An additional treatment (P/Ca-Phy-Coc±) was implemented which contained the coccidiostat in phase 1, but not in phase 2. This treatment intended to study the effect of a coccidiostat fed only during the starter phase on results at the end of the experiment.

The calculated concentration of ME was 14.0 MJ/kg DM in all diets of phase 2. All phase 2 diets contained 5 g/kg TiO_2 as an indigestible marker. The experimental diets were produced by first mixing all ingredients of the respective phase with the exception of the variable ingredients. For phase 2, this premix was divided into 2 parts. Both parts were then supplemented with either limestone and monocalcium phosphate or sand, and mixed again. Each of the resulting mixtures and the phase 1 premix was then divided into 4 parts and individually supplemented with a mixture of the exogenous phytase product, the coccidiostat, and sand. Afterwards, the diets were mixed again and pelleted without using steam conditioning at a pelleting temperature below 80 °C, which was checked continuously. Pellet diameter was 2 mm for the phase 1 mixtures, and 3 mm for the phase 2 mixtures. Representative samples of each diet were taken, pulverized by a vibrating cup mill (PULVERISETTE 9, Fritsch GmbH, Idar-Oberstein, Germany) and analyzed. Intended concentrations of P, Ca, phytase and coccidiostat were confirmed by analysis (Table 5).

Table 4 Ingredient composition of the diets and calculated concentrations

Ingredient, g/kg	Phase 1 (d 1-10)	Phase 2 (d 10-24/25)	
	P/Ca+[1]	P/Ca-[1]	P/Ca+[1]
Corn	550	575	575
Soybean Meal	370	350	350
Soy Crude Oil	30	30	30
Monocalcium Phosphate	17	-	11
Limestone	16	11	16
Sand	-	16	-
Vitamin Premix[2]	2	2	2
Mineral Premix[3]	0.5	0.5	0.5
DL-Methionine	2	3.5	3.5
Sodium Bicarbonate	3	3	3
Sodium Chloride	1.5	1	1
Choline Chloride	2	2	2
TiO_2	5	5	5
Calculated composition, g/kg DM			
Crude Protein	237	230	230
Total Phosphorus (tP)	8.5	4.2	7.0
Calcium	11.5	6.5	10.4
Ca:tP	1.3	1.5	1.5

[1]Includes treatments Phy-Coc-, Phy-Coc+, Phy+Coc-, and Phy+Coc+, where Phy- = 0 and Phy+ = 1500 FTU phytase/kg, Coc- = 0 and Coc+ = 50 mg/kg of Narasin and Nicarbazin each in exchange for sand

[2]Vitamin premix (Miavit GmbH, Essen, Germany), provided per kg of complete diet: 10 000 IU vitamin A, 3000 IU vitamin D3, 30 mg vitamin E, 2.4 mg vitamin K3, 100 mcg biotin, 1 mg folic acid, 3 mg vitamin B1, 6 mg vitamin B2, 6 mg vitamin B6, 30 mcg vitamin B12, 50 mg nicotinamide, 14 mg calcium-D-pantothenat

[3]Trace element premix (Gelamin Gesellschaft für Tierernährung mbH, Memmingen, Germany), provided per kg of complete diet: 25 mg calcium from carbonate, 80 mg manganese from manganese-(II)-oxide, 60 mg zinc from zinc-oxide, 25 mg iron from ferrous-(II)-sulphate monohydrate, 7.5 mg copper from cupric-(II)-sulphate pentahydrate, 0.6 mg iodine from calcium iodate, 0.2 mg selenium from sodium selenite

Sampling and measurements

Animals and feeds were weighed on a pen basis before placement, on d 10, and before slaughter to calculate ADFI, ADG and G:F. To standardize intestinal fill, feed was deprived 2 h before slaughtering followed by 1 h *ad libitum* access to feed. On d 24 (36 pens; 4 pens from each treatment) and on d 25 (27 pens; 3 pens from each treatment) animals were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2. For blood samples, two randomly chosen birds per pen were killed by decapitation. The trunk blood was collected in tubes containing clot activator for serum samples or sodium fluoride and heparin for plasma samples. Blood samples were then centrifuged for 10 min at 2, 500 × g to separate the plasma. The remaining 8 anaesthetized birds of each pen were euthanized by CO_2 asphyxiation. The right foot of each bird was removed and frozen at -20 °C for bone ash analyses. Digesta from the crop and the terminal part of the ileum, defined as the last two thirds of the section between Meckel´s diverticulum and 2 cm prior the ileo-caeco-colonic junction, were collected and pooled on a pen basis. The crop was clamped with an arterial clamp to prevent emptying, then opened and upended. Crop digesta was gently removed with a spatula without scraping the mucosa, mixed, and pH was measured using a spear-tip electrode (InLab® Solids; Mettler-Toledo, Gießen, Germany) and a subsample was collected into tubes for microbiota analysis. From the terminal ileum, approximately 2 cm was taken from each bird for the microbiota

Table 5 Analyzed composition of the experimental diets

	P g/kg DM	Ca g/kg DM	Phytase FTU/kg	Narasin mg/kg	Nicarbazin mg/kg	CP g/kg DM	*Myo*-Inositol g/kg DM	Ins(1,2,3,4,5)P_5 µmol/g DM	Ins(1,2,4,5,6)P_5 µmol/g DM	InsP_6 µmol/g DM
					Phase 1					
P/Ca+Phy-Coc-	8.22	11.32	<50	<1	<1	23.5	0.2	0.6	1.1	15.7
P/Ca+Phy-Coc+	8.23	11.39	<50	59	51	24.6	0.2	0.5	1.1	15.8
P/Ca+Phy+Coc-	8.60	11.94	1460	<1	<1	23.8	0.2	0.5	1.0	14.9
P/Ca+Phy+Coc+	8.75	11.53	1570	56	51	24.1	0.2	0.5	1.0	15.0
					Phase 2					
P/Ca-Phy-Coc-	4.17	6.53	<50	<1	<1	23.2	0.2	0.5	1.0	15.1
P/Ca-Phy-Coc+	4.18	6.42	<50	59	47	23.2	0.2	0.5	1.1	15.2
P/Ca-Phy+Coc-	4.19	6.48	1520	<1	<1	23.2	0.2	0.5	1.1	15.2
P/Ca-Phy+Coc+	4.20	6.42	1580	60	45	23.3	0.2	0.6	1.2	16.2
P/Ca+Phy-Coc-	7.13	10.86	<50	<1	<1	23.3	0.2	0.5	1.0	15.3
P/Ca+Phy-Coc+	6.84	10.34	<50	56	47	23.1	0.2	0.5	1.0	15.1
P/Ca+Phy+Coc-	6.61	10.11	1600	<1	<1	23.3	0.2	0.5	1.0	15.6
P/Ca+Phy+Coc+	6.57	10.02	1500	60	50	23.6	0.2	0.5	1.0	15.2

analysis. These parts were cut lengthwise, digesta was gently removed with a spatula without scraping the mucosa, pooled and mixed, and pH was measured. Digesta samples for microbiota analysis were immediately stored on ice and later frozen at -80 °C until further analysis. The other parts of the terminal ileum were flushed with cold double-distilled water. Digesta samples not determined for microbiota analysis were immediately frozen at −20 °C, freeze-dried, and pulverized. Pulverized samples were stored in airtight containers until further analysis.

Chemical analyses

Feed samples were analyzed for DM according to the official methods in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA), method no. 3.1) [52]. Pulverized feed and ileum digesta samples were analyzed for CP using VDLUFA method no. 4.1.1. [52] and for P, Ca, and TiO_2 using the modified sulfuric and nitric acid wet digestion method of Boguhn et al. [53]. Measurements were done using inductively coupled plasma optical emission spectrometry, described in detail by Zeller et al. [2].

The extraction and measurement of Ins$P_{3–6}$ isomers in feed and digesta were carried out using the method of Zeller et al. [2] with slight modifications, described in detail by Sommerfeld et al. [4]. Using this methodology, separation of enantiomers is not possible. Hence, the presentation of results does not distinguish between D- and L-form. Filtrates were analyzed using high-performance ion chromatography and UV detection at 290 nm in an ICS-3000 system (Dionex, Idstein, Germany). Since some specific InsP_3 isomer standards were not available, theses isomers could not be identified. A clear discrimination between the isomers Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(2,4,5)P_3 was not possible due to co-elution. Thus, the term InsP_{3x} will be used for these isomers with unknown proportions.

For analysis of MI, feed and digesta were analyzed according to Sommerfeld et al. [4]. Measurements were done using an Agilent 5977A gas chromatograph/mass spectrometer (Waldbronn, Germany) with deuterated MI used as an internal standard.

Alkaline phosphatase, Ca and P_i in blood serum were analyzed at the IDEXX BioResearch Vet Med Labor GmbH (Ludwigsburg, Germany) with a Beckman Olympus AU480. P_i was measured as phosphomolybdate complex and Ca according to the Arsenazo method. For ALP, the method of the International Federation of Clinical Chemistry with 2-amino-2-methyl-1-propanol buffer was used [54].

The right foot of each bird was detached at the *articulatio intertarsalis* including skin, claws, and all adhering tissues after defrosting. Feet were washed with distilled water, first dried for 48 h at 60 °C in a convection oven (VL 115, VWR International GmbH, Darmstadt, Germany), then for 72 h at 103 °C. Subsequently, they were ashed in a muffle furnace (Nabertherm L 40/11/B170, Nabertherm GmbH, Bremen, Germany) for 48 h at 600 °C.

Feed samples were analyzed for phytase activity by AB Vista Laboratories (Ystrad Mynach, UK) using the analytical method of the supplier (pH 4.5, 60 °C) followed by transferring the results to the commonly used FTU by a validated transfer factor.

For analysis of coccidiostat, feed samples were measured with high performance liquid chromatography at the LUFA Speyer (Germany). Narasin was analyzed according to VDLUFA method no. 14.22.1 [52], and

Nicarbazin according to method DIN EN 15782 of the European Committee for Standardization [55].

DNA extraction, illumina amplicon sequencing and data analysis

DNA from crop and ileum digesta samples was extracted with the commercial DNA extraction kit FastDNA™ Spin Kit for Soil (MP Biomedicals LLC, Solon, OH, USA) following the manufacturer's instructions. DNA was quantified with a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20 °C. Illumina library was prepared according to Kaewtapee et al. [56]. In brief, the V1-2 region of the 16S rRNA gene was amplified in a two-step polymerase-chain reaction (PCR). One microliter of DNA was used as template in the first PCR, where the forward primer contains a six-nucleotide barcode and both primers have sequences complementary to the Illumina adapters. Subsequent, one microliter of the first PCR product was used in a second PCR following the same conditions, where both primers were complemented to the sequences of Illumina multiplexing and Illumina index primers [57]. The amplicons were verified in an agarose gel electrophoresis with a 2 % agarose gel (ROTH Bioenzyme). Purification and normalization were done through the SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA, USA). The amplicons were pooled per Index and a second purification was performed with the MinElute PCR Purification Kit (Qiagen). Samples were sequenced using the 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform.

Raw reads were checked for quality, assembled and aligned using Mothur pipeline tool [58]. The data included 74,662 ± 3,399 sequences per sample. The UCHIME program included in Mothur pipeline was used to identify possible chimeras [59]. Reads were clustered at 97 % identity into 681 OTUs. Only OTU with an average abundance higher than 0.0001 % and a sequence length >250 bp were considered for further analysis. The closest representative was manually identified using seqmatch from the Ribosomal Database Project [60]. Sequences were submitted to the European Nucleotide Archive (study accession number PRJEB28349).

Calculations and statistical analyses

To calculate ADG and ADFI, birds' weight gain or feed consumption on a pen basis were divided by days of life of all animals in a pen. Dead animal´s weight and the pen feed consumption up to the day of occurrence were recorded and considered in the calculation of performance traits. The precaecal digestibility of P, Ca, and CP as well as the $InsP_6$ disappearance (Y) were calculated with the following equation:

$$Y\ (\%) = 100 - 100 * \left(\frac{Ti\ in\ feed}{Ti\ in\ digesta} * \frac{y\ in\ digesta}{y\ in\ feed} \right)$$

Where y is the concentration of the respective trait and Ti and y are in grams per kilogram DM.

All traits with the exception of the microbiota data were analyzed using a three-way ANOVA with the MIXED procedure of the software package SAS for Windows (version 9.3; SAS Institute Inc., Cary, NC). While treatment effects were taken as fixed, block effects were assumed as random. For each trait, heterogeneity of error variances between treatments, P/Ca-, phytase- or coccidiostat-levels and combinations of these factors were tested and the model with the smallest AIC was used. For all traits analyzed in this experiment, with the exception of blood and bone ash, samples were pooled on a pen basis and therefore the pen was determined as experimental unit. The following model was fitted:

$$Y_{ijkl} = \mu + s_l + \alpha_{il} + \beta_{jl} + \gamma_{kl} + (\alpha\beta)_{ijl} + (\alpha\gamma)_{ikl} + (\beta\gamma)_{jkl} + (\alpha\beta\gamma)_{ijkl} + \varepsilon_{ijkl}$$

Where: Y_{ijk} = observation of the response variable, μ = general effect, s_l = effect of treatment P/Ca-Phy-Coc±, α_{il} = effect of P/Ca supplementation, β_{jl} = effect of phytase supplementation, γ_{kl} = effect of coccidiostat supplementation, all possible interactions among the effects and ε_{ijkl} = residual error. A graphical check of residuals for normal distribution and homogeneity of variance was done. After finding significant effects, simple or marginal means were compared using a multiple t-test. Blood and bone ash were obtained from individual birds, so the bird was considered as the experimental unit. In this case the pen was included as random effect to the model described above. For bone ash, day of drying and ashing were additionally considered as random effects.

The sequencing dataset was statistical analysed using PRIMER software (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK) in a multivariate analysis. The dataset was first standardized by total, then comparisons between samples were done through a sample similarity matrix using the Bray-Curtis coefficient. Intersection matrix to define core microbiota was done based on the R package UpSet [61]. The community similarity structure was depicted through nMDS. Samples were represented as points in low-dimensional 2D space. A hierarchical cluster analysis was done to show the similarity between samples. PERMANOVA, a non-parametric multivariate statistical test, was used to study the significant differences and interactions of P/Ca, phytase and coccidiostat supplementation on the microbial community in crop and ileum and between the sections. The similarity percentage analysis (SIMPER) identified the OTU contribution to the similarity among

samples within each treatment. The Shannon-Weaver index of diversity (H') was used to calculate sample diversity. Differences in the abundance of OTUs between treatments were evaluated using the unpaired Welch's t-test in Excel that is able to handle unequal variances, unequal sample sizes and non-parametric data [62]. Correlations were estimated with Pearson correlation coefficient (9999 permutations) using PRISM6 (GraphPad Software, CA). For all statistical analyses, significance was declared at $P \leq 0.05$.

Additional file

Additional file 1: Table S1. Effect of the experimental diets on InsP isomers and pH in crop digesta. **Table S2.** Effect of the experimental diets on InsP isomers, *myo*-inositol and pH in ileum digesta. **Table S3.** Effect of the experimental diets and the sampling section on the microbial composition (PERMANOVA analysis). **Table S4.** Effect of different coccidiostat treatments on performance traits of broilers. **Table S5.** Effect of different coccidiostat treatments on precaecal nutrient digestibility, $InsP_6$ disappearance and foot ash. **Table S6.** Effect of different coccidiostat treatments on blood metabolites and pH in digesta. **Figure S1.** Distribution of the Operational Taxonomic Units (OTUs). **Figure S2.** Cluster analysis for crop (A) and ileum (B) digesta samples. **Figure S3.** Non-metric multidimensional scaling plot illustrating the global bacterial community structure. **Figure S4.** Relative abundance for more abundant Operational Taxonomic Units (OTUs) in Crop (A) and ileum (B) digesta samples. (DOCX 4972 kb)

Abbreviations

ADFI: Average daily feed intake; ADG: Average daily gain; ANOVA: Analysis of variance; BW: Bodyweight; Ca: Calcium; Coc: Coccidiostat; CP: Crude protein; G:F: Gain-to feed ratio; H´: Shannon-weaver index of diversity; $InsP_6$: *Myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate); MI: *Myo*-inositol; nMDS: Non-metric multidimensional scaling plot; OTU: Operational taxonomic units; P: Phosphorus; P/Ca: Phosphorus and Calcium; PCR: Polymerase-chain reaction; Phy: Phytase; P_i: Inorganic phosphate

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Authors' contributions

Conceived and designed the experiment: SK, MR, IK, ACS. Performed the experiments: SK, RK, DBM. Animal data analysis: SK, VS, MR. Microbial data analysis: DBM, RK, ACS. Statistical analysis: SK, ACS, DBM, RK. Drafting the paper: SK, DBM. Paper revisions and final approval: SK, DBM, VS, MR, ACS, IK.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval

The trial was performed in accordance with the German Animal Welfare Legislation, approved by the Regierungspräsidium Tübingen, Germany (project no. HOH 46/17 TE).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Institut für Nutztierwissenschaften, Universität Hohenheim, 70599 Stuttgart, Germany. [2]AB Vista, 64293 Darmstadt, Germany.

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Publisher's Note

Table S1 Effect of the experimental diets on InsP isomers and pH in crop digesta[1]

Diet	$InsP_{3x}$[3]	$Ins(1,2,5,6)P_4$	$Ins(1,2,3,4,5)P_5$	$Ins(1,2,4,5,6)P_5$	$InsP_6$	pH crop digesta
	µmol/g DM					
P/Ca-Phy-Coc-	n.d.	0.2	0.6	1.1	14.8	5.1
P/Ca-Phy-Coc+	n.d.	<LOQ	0.8	1.1	14.5	5.0
P/Ca-Phy+Coc-	1.4	4.5	0.3	0.2	4.3	5.2
P/Ca-Phy+Coc+	1.4	4.1	0.2	0.2	3.1	5.0
P/Ca+Phy-Coc-	n.d.	<LOQ	0.7	1.1	15.1	5.1
P/Ca+Phy-Coc+	n.d.	<LOQ	0.7	1.1	15.0	5.1
P/Ca+Phy+Coc-	1.5	4.9	0.4	0.3	5.7	5.0
P/Ca+Phy+Coc+	1.4	4.6	0.3	0.2	4.4	4.9
P/Ca-Phy-Coc±[2]	n.d.	0.3	0.7	1.1	14.9	5.2
pooled SEM	0.18	0.24	0.04	0.04	0.85	0.13
			P-values			
P/Ca	0.939	0.049	0.152	0.139	0.182	0.728
Phytase	-	<0.001	<0.001	<0.001	<0.001	0.238
Coccidostat	0.789	0.181	1.000	0.341	0.276	0.126
P/Ca×Phy	-	-	0.020	0.891	0.438	0.188
P/Ca×Coc	0.789	0.805	0.230	0.891	0.947	0.562
Phy×Coc	-	-	0.020	0.495	0.432	0.606
P/Ca×Phy×Coc	-	-	0.230	0.891	0.911	0.949

[1] Not shown isomers were not detectable (n.d.) or not quantifiable (<LOQ) in the majority of samples; n=7 pens

[2] Additional treatment, was not part of the three-factorial analysis

[3] At least one of the following isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$

Table S2 Effect of the experimental diets on InsP isomers, *myo*-inositol and pH in ileum digesta[1]

	$InsP_{3x}$[3]	$Ins(1,5,6)P_3$	$Ins(1,2,3,4)P_4$	$Ins(1,2,5,6)P_4$	$Ins(1,2,3,4,5)P_5$	$Ins(1,2,3,4,6)P_5$	$Ins(1,2,4,5,6)P_5$	$InsP_6$	*Myo*-inositol	pH ileum digesta
Diet	µmol/g DM								g/kg DM	
P/Ca-Phy-Coc-	n.d.	0.2	0.3	n.d.	1.2	0.6	0.4	24.6	1.4	6.2
P/Ca-Phy-Coc+	n.d.	n.d.	0.2	n.d.	1.1	0.5	0.4	25.4	1.6	6.6
P/Ca-Phy+Coc-	n.d.	<LOQ	n.d.	0.6	0.8	n.d.	0.3	6.1	3.2	5.9
P/Ca-Phy+Coc+	0.2	<LOQ	n.d.	1.1	0.8	n.d.	0.3	7.5	3.6	6.7
P/Ca+Phy-Coc-	0.2	<LOQ	0.4	0.5	2.3	1.0	2.6	47.2	0.2	6.4
P/Ca+Phy-Coc+	n.d.	0.2	0.3	0.5	2.3	1.0	2.5	48.4	0.2	7.0
P/Ca+Phy+Coc-	2.9	<LOQ	0.4	5.8	2.7	n.d.	1.0	12.7	1.2	6.5
P/Ca+Phy+Coc+	3.7	0.2	0.5	7.1	2.8	n.d.	1.2	13.4	1.6	6.9
P/Ca-Phy-Coc±[2]	0.3	<LOQ	0.3	n.d.	1.2	0.5	0.4	23.8	1.2	6.3
pooled SEM	0.21	0.03	0.08	0.60	0.34	0.03	0.13	1.66	0.19	0.27
					P-values					
P/Ca	<0.001	-	0.022	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.004
Phytase	<0.001	0.560	0.334	<0.001	0.856	-	<0.001	<0.001	<0.001	0.746
Coccidostat	0.049	-	0.851	0.302	0.989	0.466	0.902	0.238	0.090	<0.001
P/Ca×Phy	-	-	-	-	0.168	-	<0.001	<0.001	0.013	0.559
P/Ca×Coc	-	-	0.679	0.343	0.944	0.466	0.838	0.957	0.766	0.660
Phy×Coc	-	-	0.598	0.313	0.900	-	0.541	0.997	0.401	0.536
P/Ca×Phy×Coc	-	-	-	-	0.967	-	0.596	0.747	0.620	0.337

[1] Not shown isomers were not detectable (n.d.) or not quantifiable (<LOQ) in the majority of samples; n=7 pens

[2] Additional treatment, was not part of the three-factorial analysis

[3] At least one of the following isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$

Table S3 Effect of the experimental diets and the sampling section on the microbial composition (PERMANOVA analysis)

A. Treatments of the 2 x 2 x 2 factorial arrangement of treatments

	Crop and Ileum	Crop	Ileum
		P-values	
Treatment	0.001	<0.001	<0.001
Section	0.001		
Treatment x Section	0.969		
P/Ca	0.055	0.195	0.201
Phytase	0.021	0.032	0.321
Coccidostat	<0.001	<0.001	<0.001
P/Ca×Phy	0.147	0.068	0.812
P/Ca×Coc	0.001	0.008	0.007
Phy×Coc	0.009	0.105	0.070
P/Ca×Phy×Coc	0.025	0.378	0.044

B. Pairwise comparisons between treatments P/Ca-Phy-Coc-, P/Ca-Phy-Coc+, and P/Ca-Phy-Coc±

	Crop	Ileum
P/Ca-Phy-Coc- vs P/Ca-Phy-Coc+	0.001	<0.001
P/Ca-Phy-Coc- vs P/Ca-Phy-Coc±	0.246	0.213
P/Ca-Phy-Coc+ vs P/Ca-Phy-Coc±	0.003	0.001

Table S4 Effect of different coccidiostat treatments on performance traits of broilers

	Phase 2 (d10-24/25, n=7 pens)			Phase 1+2 (d1-24/25, n=7 pens)			d24/25
	ADG	ADFI	G:F	ADG	ADFI	G:F	BW
	g/d	g/d	g/g	g/d	g/d	g/g	g
P/Ca-Phy-Coc-	35[b]	50[b]	0.71	28[b]	37[b]	0.76	703[b]
P/Ca-Phy-Coc+	40[a]	56[a]	0.72	32[a]	41[a]	0.77	785[a]
P/Ca-Phy-Coc+/-	37[ab]	52[ab]	0.71	30[ab]	39[ab]	0.77	752[ab]

[a–f] Means within a column not showing a common superscript differ ($P \leq 0.05$)

Table S5 Effect of different coccidiostat treatments on precaecal nutrient digestibility, $InsP_6$ disappearance[1] and foot ash[2]

	P digestibility	Ca digestibility	CP digestibility	Crop $InsP_6$ disappearance	Ileum $InsP_6$ disappearance	*Myo*-Inositol	Foot ash	Foot ash
	%	%	%	%	%	g/kg DM	mg	% of DM
P/Ca-Phy-Coc-	47.1	64.0	77.6	-1.7[b]	47.7	1.4[ab]	568[b]	9.4[b]
P/Ca-Phy-Coc+	48.0	61.1	77.5	4.5[a]	48.6	1.6[a]	639[a]	9.9[a]
P/Ca-Phy-Coc+/-	47.3	63.1	76.5	-0.1[b]	50.3	1.2[b]	606[b]	9.5[b]

[1] n=7 pens

[2] n=70 birds

[a–f] Means within a column not showing a common superscript differ ($P \leq 0.05$)

Table S6 Effect of different coccidiostat treatments on blood metabolites and pH in digesta

	P_i	Ca	ALP	*Myo*-Inositol	Crop pH	Ileum pH
	mmol/l	mmol/l	U/l	µmol/ml		
P/Ca-Phy-Coc-	1.2[ab]	3.1[ab]	9239[a]	0.33	5.1	6.2
P/Ca-Phy-Coc+	1.4[a]	3.3[a]	6525[b]	0.31	5.0	6.6
P/Ca-Phy-Coc+/-	1.2[b]	2.9[b]	8457[ab]	0.30	5.2	6.3

[1] P_i: inorganic phosphate, Ca: calcium and ALP: alkaline phosphatase in blood serum; *myo*-inositol in blood plasma; n=14 birds; pH values: n=7 pens

[a–f] Means within a column not showing a common superscript differ ($P \leq 0.05$)

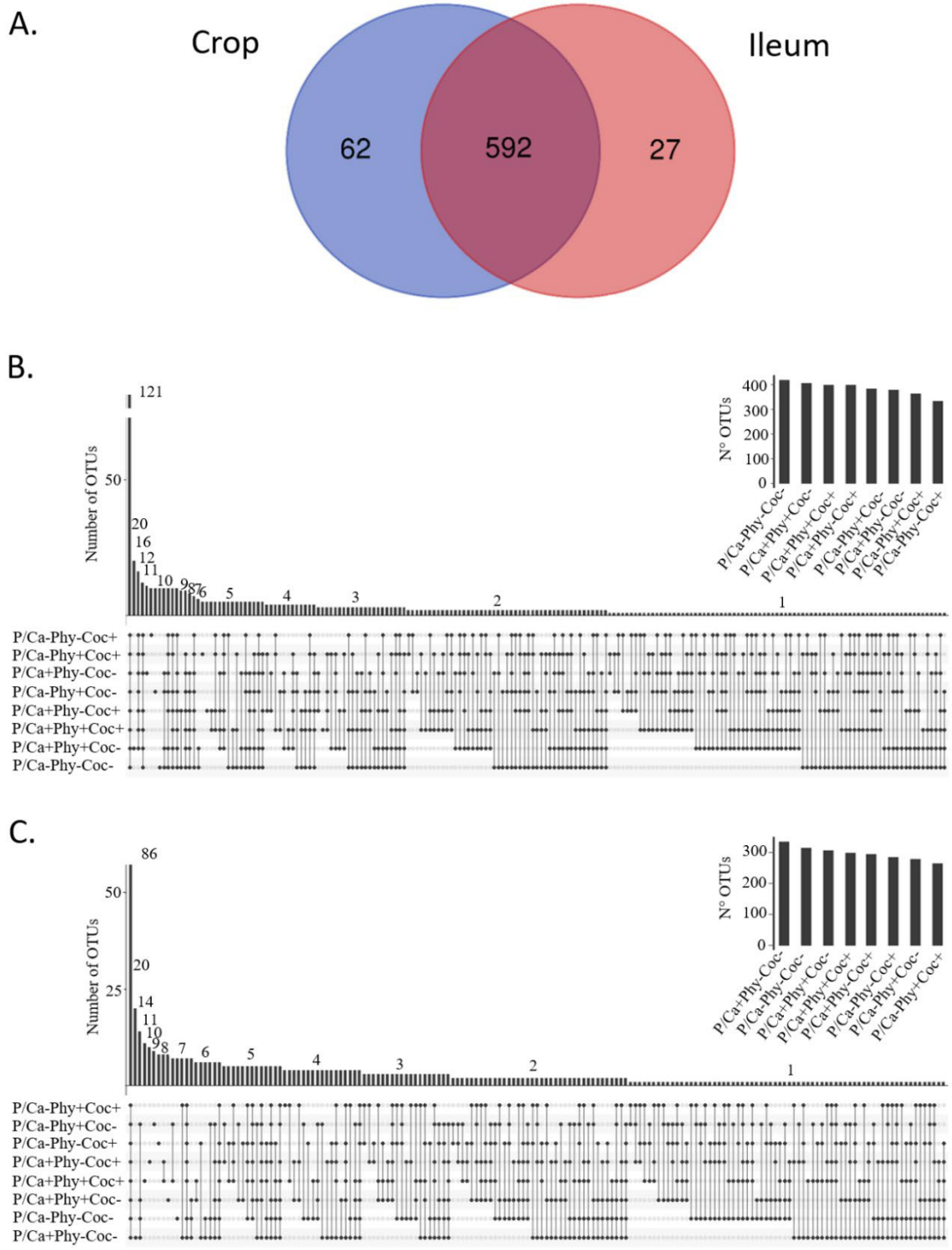

Figure S1 Distribution of the Operational Taxonomic Units (OTUs). Venn diagram showing the intersection between the crop and ileum (A). Matrix layout to show the number of OTUs for the core microbiota at Operational Taxonomic Units level found in the crop (B) and ileum (C)

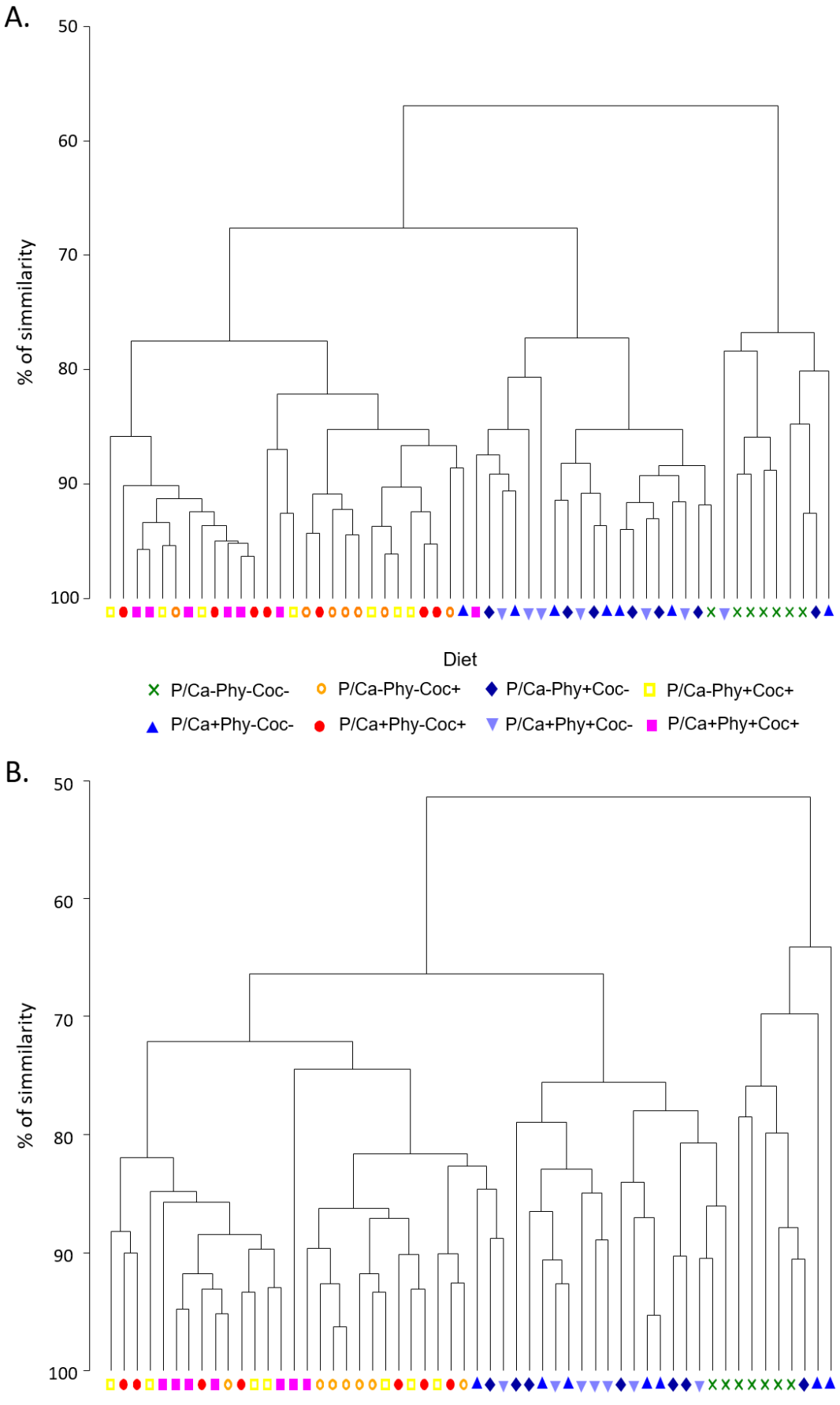

Figure S2 Cluster analysis for crop (A) and ileum (B) digesta samples

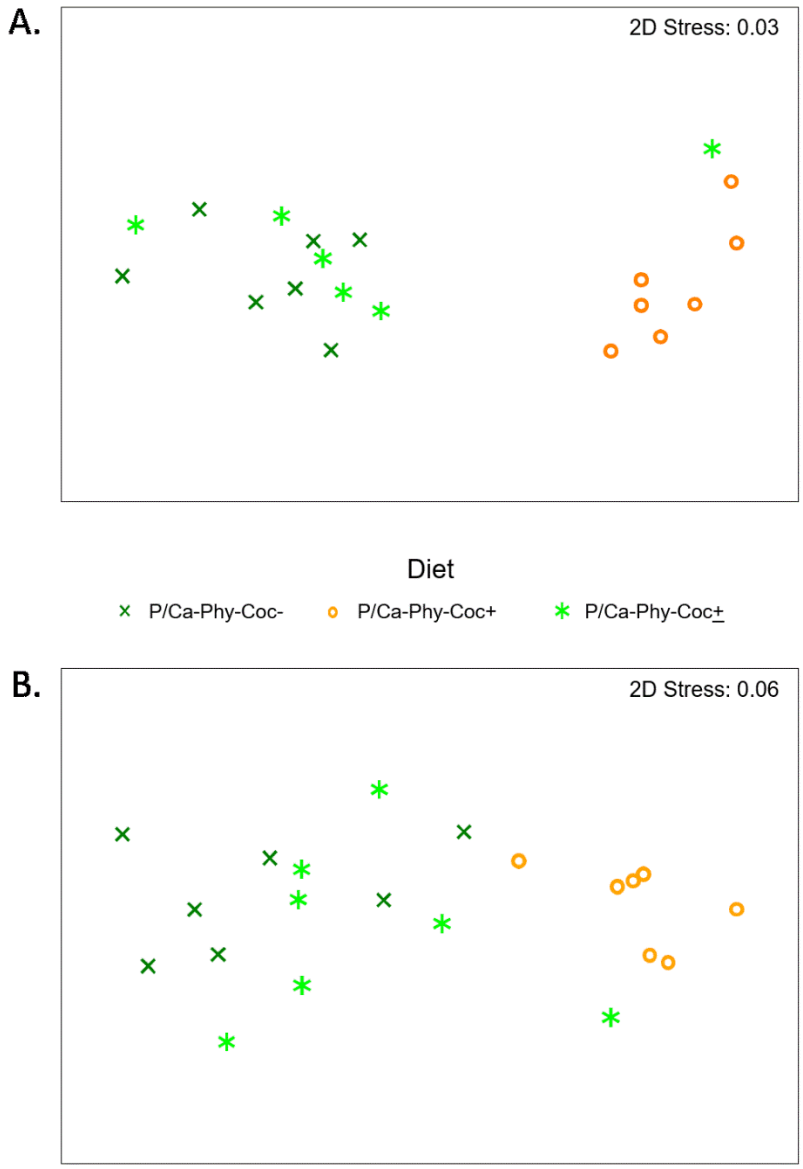

Figure S3 Non-metric multi-dimensional scaling plot illustrating the global bacterial community structure. Dietary treatments are shown based on their replicates obtained for the digesta samples of crop (A) and ileum (B). The symbols represent one pooled sample from each pen comprising all Operational Taxonomic Units

5 SUMMARY

Phosphorus (P) is an essential element that is crucial for various metabolic processes in the body of animals and humans. To keep the animals healthy and to obtain food products rich in nutrients, an adequate P supply is indispensable. Plant feedstuffs, the main components of poultry diets, contain P in a form that is only partially available to poultry. For this reason, poultry diets are often supplemented with mineral P. However, global rock phosphate reserves, where mineral P is mined from, are limited. Additionally, excessive P supply should also be avoided because of the environmental impact of P accumulation in the soil. Consequently, P supply not exceeding the requirements of poultry is essential to ensure animal wellbeing and to protect the environment. In order to feed diets with adequate concentrations of P, it is necessary to have suitable approaches for the determination of available P in the animal. The availability of P varies widely between feed components and it is also influenced by feed supplements and other factors. Bone ash analysis is an often-used tool to evaluate the relative bioavailability of P since a high amount of P is stored in the bones. A standard assay for bone ash analyses has never been agreed on. Therefore, many different approaches are described in the literature with an unknown impact on the results of P bioavailability studies.

The main objective of the present thesis was to examine the suitability of bone ash data for the evaluation of available P in poultry with emphasis on methodological aspects. Therefore, different studies with broiler chickens and Japanese quail were conducted. The experiments comprised various aspects related to P availability in poultry. The effect of feed supplements in the form of phytase products, *myo*-inositol and a coccidiostat were evaluated. Furthermore, quantitative genetic analyses were performed. All experiments had in common that tibiotarsus (tibia) or foot ash data or both were used for the examination of the relative bioavailability of P. Based on the data that accrued during the studies described in the four manuscripts of this thesis, comprehensive methodological analyses were performed.

The tibia and foot were compared regarding their appropriateness as a trait for the evaluation of the relative bioavailability of P by using bone ash data. The relationship between the two traits was investigated, as well as the relationship between foot or tibia ash and quantitative P measurements. Additionally, P concentration in the ash of both bone fractions was analysed and compared. Results indicated only minor differences between tibia and foot ash data. No clear preference for one of them could be deduced from the data.

The left and right feet of broiler chickens were compared in terms of both ash concentration and total ash amount. Significant differences between the two feet of the same animal were detected

for both traits. Consequently, not only the choice of the bone fraction but also of the body side should be considered when sampling for bone ash data.

Ash data are mostly expressed as a concentration of the dry matter content of the bone. Also possible is the use of the absolute ash amount. The relationship of both ways of expression with traits of quantitative P measurements was analysed by using correlation coefficients and regression analyses. Results showed that the absolute ash amount was at least as suitable as ash concentration but has the advantage that it is easier to determine.

Possible selection procedures for animals for bone ash analyses were simulated with data from two of the experiments. Often it is not possible to use all animals involved in an experiment for bone ash analyses. Therefore, the influence of sampling frequency and selection method on the outcome of P availability studies was evaluated. Results indicated that the number and selection method of animals for bone ash data might influence the results. However, it was not possible to recommend a specific selection method based on the obtained results.

Estimates of heritability and genetic correlations showed the suitability of bone ash data as a proxy trait for P efficiency breeding of poultry. The absolute amount of bone ash data appeared to be most promising for this purpose.

Bone ash data are a very useful and easy to determine trait to estimate the relative bioavailability of P. However, investigations performed in this thesis showed the importance of a careful selection of methods. A standardised assay would be helpful to obtain meaningful and more comparable estimates of relative P bioavailability.

6 ZUSAMMENFASSUNG

Phosphor (P) ist für Tiere und Menschen ein essenzielles Element, das für zahlreiche Stoffwechselvorgänge im Körper benötigt wird. Eine ausreichende Versorgung mit P ist unabdingbar, um Tiere gesund zu erhalten und um nährstoffreiche Lebensmittel zu erzeugen. Der Hauptbestandteil von Geflügelrationen sind pflanzliche Futtermittel. Diese enthalten P in einer Form, die nur teilweise für Geflügel nutzbar ist. Deshalb werden Geflügelrationen meist mit mineralischem P ergänzt. Dieser mineralische P wird aus Gesteinsphosphaten gewonnen, deren global vorhandene Ressourcen begrenzt sind. Zusätzlich ist auch eine übermäßige Versorgung der Tiere mit P zu vermeiden, da große Mengen an P-Ausscheidungen negative Auswirkungen auf die Umwelt durch die Anreicherung im Boden haben. Daher ist eine Versorgung der Tiere nahe an ihrem Bedarf notwendig, um ihre Gesundheit und die Umwelt zu schützen. Für eine Fütterung der Tiere nahe an ihrem P-Bedarf ist es notwendig, geeignete Verfahren für die Bestimmung des im Tier verfügbaren P anwenden zu können. Der Anteil an verfügbarem P variiert stark zwischen verschiedenen Futterbestandteilen und wird von Futterzusätzen und anderen Faktoren beeinflusst. Eine weit verbreitete Möglichkeit hierfür ist die Ermittlung der relativen Bioverfügbarkeit von P anhand von Knochenasche-Daten. Ermöglicht wird dieses Verfahren durch die große Menge an P, die in den Knochen eingelagert ist. Für die Verwendung von Knochenasche gibt es allerdings kein standardisiertes Vorgehen, was dazu führt, dass viele verschiedene Methoden in der Literatur beschrieben sind. Der Einfluss von verschiedenen Vorgehensweisen auf die Ergebnisse der Arbeiten, die die relative Bioverfügbarkeit von P untersuchen, ist unbekannt.

Das Hauptziel der vorliegenden Arbeit war es, die Eignung von Knochenasche-Daten für die Ermittlung des verfügbaren P bei Geflügel zu überprüfen. Der Schwerpunkt lag dabei auf methodischen Gesichtspunkten. Für diesen Zweck wurden verschiedene Versuche mit Broilern und Japanischen Wachteln durchgeführt. Diese beinhalteten verschiedene Aspekte der Verfügbarkeit von P bei Geflügel. Analysiert wurden die Auswirkungen von verschiedenen Futterzusätzen wie Phytasen, *myo*-Inositol und einem Kokzidiostatikum. Außerdem wurde eine quantitative genetische Auswertung durchgeführt. Die Gemeinsamkeit aller Versuche bestand darin, dass Tibiotarsus- (Tibia) oder Fußasche oder die Asche beider Knochenfraktionen für die Bestimmung der relativen Bioverfügbarkeit von P verwendet wurden. Anhand der Daten, die in den vier Manuskripten dieser Dissertation beschrieben wurden, wurden umfassende methodische Auswertungen durchgeführt.

Die beiden Knochenasche-Fraktionen Tibia und Fuß wurden im Hinblick auf ihre Eignung als Merkmal für die Beurteilung der relativen P-Bioverfügbarkeit verglichen. Dabei wurde die

Beziehung der beiden Knochenasche-Fraktionen zueinander ebenso betrachtet wie ihre Beziehung zu Merkmalen der quantitativen P-Bewertung. Auch der P-Gehalt in der Asche des jeweiligen Knochenfraktion wurde analysiert und verglichen. Dabei zeigte sich, dass Tibia und Fuß sich in allen untersuchten Aspekten sehr ähnlich verhielten. Eine Entscheidung zwischen der Verwendung von Tibia- und Fußasche kann nach aktuellem Kenntnisstand anhand der bevorzugten Probenahme getroffen werden.

Der rechte und linke Fuß von Broilern wurden sowohl im Hinblick auf ihre Aschekonzentration als auch auf die absolute Aschemenge verglichen. Dabei konnten für beide Merkmale signifikante Unterschiede zwischen den beiden Füßen desselben Tieres festgestellt werden. Somit sollte nicht nur die Wahl des Körperteils, sondern auch die der Körperseite bei der Gewinnung von Probenmaterial für Aschedaten berücksichtigt werden.

In der Literatur werden Aschedaten meist als Konzentration in Bezug auf die Trockenmasse der Tibia oder des Fußes angegeben. Es ist jedoch auch die Verwendung der absoluten Aschemenge möglich. Das Verhältnis der beiden Varianten mit Merkmalen der quantitativen P-Bewertung wurde mit Hilfe von Korrelationskoeffizienten und Regressionsanalysen verglichen. Dabei zeigte sich, dass die absolute Aschemenge mindestens ebenso gut geeignet ist wie die Aschekonzentration, jedoch den Vorteil der einfacheren Bestimmung hat.

Anhand der Daten aus zwei der beschriebenen Versuche wurden mögliche Auswahlverfahren von Tieren für Knochenasche-Analysen simuliert. Häufig ist es nicht möglich, alle Tiere, die an einem Versuch beteiligt sind, für die Analyse von Knochenasche zu verwenden. Daher sollte der Einfluss von Anzahl und Auswahlverfahren der Tiere auf das Ergebnis von Versuchen, die die Bewertung von verfügbarem P als Ziel haben, ausgewertet werden. Die Ergebnisse zeigten, dass die Anzahl und Auswahlmethode der Tiere die Ergebnisse dieser Versuche beeinflussen können. Es war anhand der Daten jedoch nicht möglich, ein spezifisches Auswahlverfahren zu empfehlen.

Die Berechnung von Heritabilitäten und genetischen Korrelationen zeigte die grundsätzliche Eignung von Knochenasche-Daten als Hilfsmerkmal für die Züchtung von Geflügel mit einer höheren P-Effizienz. Als besonders vielversprechend erwies sich dabei die absolute Menge an Fußasche.

Knochenasche-Daten sind ein sehr nützliches und einfach zu ermittelndes Merkmal für die Ermittlung der relativen Bioverfügbarkeit von P. Die Untersuchungen, die im Rahmen dieser Dissertation durchgeführt wurden, zeigen jedoch die Bedeutung einer gewissenhaften Methodenauswahl. Ein standardisiertes Verfahren wäre hilfreich, um aussagekräftige und besser vergleichbare Schätzungen der relativen Bioverfügbarkeit von P zu erhalten.

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CURRICULUM VITAE

PERSONAL DETAILS

Name:	Susanne Künzel
Date of birth:	06.05.1989
Place of birth:	Crailsheim, Germany

EDUCATION

Since 10/2015	Doctoral student, Institute of Animal Science, Department of Animal Nutrition, University of Hohenheim, Germany
10/2012 – 05/2015	Master´s Program in Agricultural Biology, Major: Farm Animal Biology, University of Hohenheim, Germany Qualification gained: Master of Science
10/2009 – 09/2012	Bachelor´s Program in Agricultural Biology, Profile: Applied Farm Animal Biology, University of Hohenheim, Germany Qualification gained: Bachelor of Science
09/1999 – 07/2008	Albert-Schweitzer-Gymnasium Crailsheim, Germany Qualification gained: Abitur

EXPERIENCE

Since 02/2019	Scientific staff, Institute of Animal Science, Department of Animal Nutrition, University of Hohenheim, Germany
07/2015 – 02/2019	Research assistant, Institute of Animal Science, Department of Animal Nutrition, University of Hohenheim, Germany
08/2014 – 10/2014 + 05/2015	Student assistant, Institute of Animal Science, Department of Animal Nutrition, University of Hohenheim, Germany
04/2014 – 06/2014	Internship at the Institute of Animal Nutrition, Friedrich-Loeffler Institute, Braunschweig, Germany
11/2013 – 12/2013	Internship at Equestrian Magazine St.Georg, Hamburg, Germany
08/2011 – 09/2011	Internship at Birkhof stud, Donzdorf, Germany
09/2008 – 08/2009	Voluntary Social Year, Sonnenhof e.V., Schwäbisch Hall, Germany

Hohenheim, 17.11.2020

Susanne Künzel

DECLARATION IN LIEU OF AN OATH ON INDEPENDENT WORK

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

"Bone ash data in the context of phosphorus and phytase evaluation in poultry"

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Hohenheim, 17.11.2020

Susanne Künzel